# DOES *NEOSPORA CANINUM* CAUSE DEATH BY MULTIPLYING UNCONTROLLABLY IN AN IMMUNOLOGICALLY IMMATURE FOETUS?

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

Elizabeth Helen Gibney

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**Does *Neospora caninum* cause death by multiplying uncontrollably in an immunologically immature foetus?**

Elizabeth Helen Gibney

## ABSTRACT

The last two decades have seen the emergence of the protozoan parasite *Neospora caninum* as the most frequently diagnosed cause of bovine abortion in the UK. The polymerase chain reaction (PCR) is a commonly used tool for molecular diagnosis. In chapter 2, the relative diagnostic sensitivity of two PCR protocols routinely used within our laboratory for the detection of *N. caninum* in tissues from aborted foetuses was determined. Method one was a nested PCR method based on the internal transcribed spacer 1 region of the rRNA sequence and was consistently more sensitive than method two, a single step PCR designed to amplify a fragment of Nc5 *N. caninum*-specific genomic DNA, in our laboratory environment. Method one was therefore used to analyse samples for the rest of the experiment.

It is not fully understood why some infected cattle abort, but previous studies have shown that abortion is more likely if transplacental spread of the parasite occurs early in gestation. Bovine foetal immunocompetence develops gradually during the second half of gestation, and it has been suggested that the immunocompetence of the foetus at the time of infection may determine its ability to control parasitaemia and survive. To test this hypothesis in chapter 3, we compared the distribution of parasites and the histopathological changes in the placenta and foetus following experimental infection of cattle with *N. caninum* in early and late gestation. In early gestation, following foetal death, *N. caninum* DNA was detected and evidence of widespread parasite infiltration was demonstrated immunohistologically in the placenta. This was associated with extensive focal epithelial necrosis, serum leakage and a moderate maternal predominantly CD4+ mononuclear cell interstitial inflammatory response. Widespread parasite infiltration was also evident in the foetus, with parasites in most tissues, often associated with necrosis. In late gestation, *N. caninum* DNA was detected sporadically but parasites were not evident immunohistologically in the placenta. Small foci of necrosis were seen occasionally, with a mild CD4+ and CD8+ mononuclear cell interstitial inflammation. Detection of *N. caninum* DNA in the foetus was sporadic and parasites were demonstrated immunohistologically in brain and spinal cord only, with an associated non-suppurative inflammatory response.

We further investigated the pathogenesis of abortion in chapter 4, where we monitored ten chronically infected cattle throughout gestation and parasite recrudescence was pinpointed in 9/10 via a sharp rise in *N. caninum*-specific antibodies. In all cases foetal death did not occur and animals were euthanased 1-5 weeks after the antibody rise. The placentae demonstrated widespread focal necrosis, with sporadic detection of parasites and a mild to moderate lymphocyte dominated maternal interstitial infiltration. Lesions were detected in 7/10 of the foetuses and included non-suppurative myositis, myocarditis, encephalitis and myelitis. Parasite DNA was detected in 7/10 foetuses and was most commonly detected in the brain.

This data is consistent with an uncontrolled parasitaemia in an immunologically immature foetus, which could, via multiparenchymal necrosis, lead to foetal death. Additionally, the widespread necrosis and inflammation observed in the placenta after infection early in gestation could, through hypoxia and/or malnutrition of the foetus, be a contributing factor to abortion. Finally, the data in chapter 4 reveals that the parasite can be detected in the placenta and foetus, in some cases with associated necrosis and/or inflammation, without causing foetal death and this has important implications for the diagnosis of *Neospora*-associated abortions.

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# ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| AI | artificial insemination |
| BVDV | bovine viral diarrhoea virus |
| °C | degree celcius |
| d | day |
| DNA | deoxyribonucleic acid |
| dpi | days post infection |
| ELISA | enzyme-linked-immunosorbent assay |
| G | gravity |
| h | hour |
| IBR | infectious bovine rhinotracheitis |
| IFAT | immunofluorescent antibody test |
| Ig | immunoglobulin |
| kg | kilogram |
| L | litre |
| µg | microgram |
| mg | milligram |
| ml | millilitre |
| min | minute |
| NAA | *Neospora caninum*-associated-abortion |
| NRS | normal rabbit serum |
| OD | optical density |
| OR | odds ratio |
| % | percent |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PP | percent positivity |
| rDNA | ribosomal deoxyribonucleic acid |
| SD | standard deviation |
| TBS | Tris buffered saline |
| VLA | Veterinary Laboratories Agency |

# Chapter one

# Literature review

**History**

The last 20 years have seen the emergence of *Neospora caninum* as a protozoan parasite of significant veterinary importance causing both bovine abortion and neurological disease in dogs. The first reports were of an unidentified protozoan parasite causing encephalitis and myositis in dogs in Norway in 1984. The parasite, although resembling *Toxoplasma gondii* morphologically, did not react with antibodies to *T. gondii* (Bjerkas et al., 1984). In 1988, Dubey et al. described further canine cases with menigoencephalomyelitis, myositis and ulcerative dermatitis (Dubey et al., 1988a). The parasite was isolated in cell culture, Koch's postulates were fulfilled and the agent named *Neospora caninum* (Dubey et al., 1988b). Retrospective studies have since shown that *N. caninum* has been responsible for clinical disease in dogs as early as 1957 but was misdiagnosed as *Toxoplasma* (Dubey et al., 1990).

Similarly in cattle, an unidentified protozoan parasite was reported as a cause of encephalitis and myocarditis in aborted foetuses (Thilsted & Dubey, 1989). Again, the parasite resembled *T. gondii*, but antibodies to *Toxoplasma* were not found in foetal fluids. The parasite was identified as *N. caninum* using an immunoperoxidase staining technique (Lindsay & Dubey, 1989). Retrospective studies on aborted foetuses from the late 1980's confirmed that *N. caninum* was a major cause of bovine abortion in California (Barr et al., 1990, 1991, Anderson et al., 1991). Field investigations worldwide identified *N. caninum* as a causative agent for abortion in the USA (Nietfeld et al., 1992, Yaegar et al., 1994), New Zealand (Thornton et al., 1994), Australia (Obendorf et al., 1995) and South Africa (Jardine & Wells 1995). *Neospora* was first identified in aborted bovine

foetuses in the UK in 1993 (Otter et al., 1995). *Neospora*-associated abortions in cattle have since been reported throughout the world (Dubey & Lindsay, 1996).

*Neospora* has been classified in the family Sarcocystidae as a sister group to *Toxoplasma* in the phylum Apicomplexa (Dubey & Lindsay, 1996). Studies have shown that the bovine and canine isolates represent the same species. No morphological differences have been found among *N. caninum* isolates obtained from cattle and dogs (Holmdahl et al., 1997). However, genetic and biological diversity has been observed among six *N. caninum* isolates from cattle and dogs (Schock et al., 2001). In this study, genetic analysis of the isolates by RAPD-PCR produced a large number of polymorphic markers that revealed significant genetic variation. *Neospora hughesi,* isolated from the central nervous system of an adult horse in California in 1998, was confirmed as a species distinct from *N. caninum* by molecular analysis of the ITS1 region, revealing seven nucleotide base differences between the species (Marsh et al., 1996).

**Lifecycle**

The lifecycle of the parasite involves the dog and other canids as the definitive host and a variety of intermediate hosts including cattle, sheep and horses (McAllister et al., 1998). There are three parasite stages in the *Neospora* lifecycle.

Oocysts, the most recently identified stage, are produced by sexual replication in the intestines and expelled in the faeces of the definitive host, following ingestion of bradyzoites (McAllister et al., 1998). Sporulation of the oocyst takes

48 hours under laboratory conditions (McAllister et al., 1998) to the sporulated oocyst, considered to be the main route for postnatal infection to the intermediate host (Wouda et al., 1999a).

Oocysts have been demonstrated in the faeces of *Canis familiaris* (domestic dog) and *Canis latrans* (coyotes) after experimental infection (McAllister et al., 1998, Lindsay et al., 1999, Dijkstra et al., 2001a, Schares et al., 2001a, Gondim et al., 2002, 2004 and Rodrigues et al., 2004) and have been identified in the faeces of naturally infected dogs (Basso et al., 2001a, Slapeta et al., 2002 and McGarry et al., 2003). Oocysts of *N. caninum* have been found to be excreted in relatively low numbers compared to *T. gondii* oocysts excreted by infected cats (Dubey, 2002). Of epidemiological significance are reports that dogs shed higher numbers of oocysts when fed muscle tissues of intermediate hosts (Schares et al., 2001a) and infected cattle placentae (Dijkstra et al., 2001a).

Bradyzoites are the slowly multiplying stage of the parasite, contained within tissue cysts in a wide range of intermediate hosts, including cattle. These tissue cysts are found primarily in nervous tissue, but have also been described in bovine placenta (Shivaprasad et al., 1989) and skeletal muscle of dogs and cattle (Peters et al., 2001). The fact that these tissue cysts are HCl-pepsin resistant (Lindsay & Dubey, 1996) supports the theory of a carnivorous definitive host.

Thirdly, tachyzoites are the extra-intestinal actively multiplying stage. They penetrate and rupture host cells following replication by endodyogeny. This stage can be transmitted transplacentally to the foetus in pregnant cattle (Dubey and

Lindsay, 1996). Tachyzoites are most commonly found in the brain, heart and liver of bovine foetuses (Barr et al., 1991, Wouda et al., 1997). It is assumed that, with the onset of the host immune response and the presence of other physiological factors, tachyzoites differentiate into bradyzoites and a persistent tissue cyst infection is established (Buxton et al., 2002a).

**Infection in cattle**

Seroprevalence studies indicate that *N. caninum* has a worldwide distribution and in the UK, it is estimated that 6% of the national herd is infected and around 6,000 abortions a year are attributable to neosporosis (Davison et al., 1999).

The transmission of the parasite to the foetus in pregnant cattle can result in abortion, or the birth of calves that are persistently infected. This transplacental spread can be endogenous, resulting from the recrudescence of bradyzoite cysts in chronically infected cows, or exogenous, resulting from the ingestion of sporulated oocysts during pregnancy (Trees and Williams 2005). *N. caninum* is the most frequently diagnosed abortifacient in dairy cattle in the UK and is a cause of abortion worldwide (VLA Surveillance Report 2006; Dubey, 1999). Herd abortion patterns tend to be sporadic or endemic but occasionally epidemics occur (Nietfeld et al., 1992; Yaeger et al., 1994; Thornton et al., 1994; Dannatt et al., 1995; McAllister et al., 1996; McAllister et al., 2000; Moen et al., 1998; Patitucci et al., 1999; Schares et al., 1999a; Schares et al., 1999b; Jenkins et al., 2000; Atkinson et al., 2000; Dijkstra et al., 2001). The average gestational age of a foetus aborted due to *N. caninum* is 5 – 6 months, with a range of 3.5 - 9 months (Barr et al., 1990; Anderson et al., 1991; Anderson et al., 1995; Otter et al., 1995;

Wouda et al., 1997). Case:control studies using aborting and normally calving cow populations have shown that seropositive cows have between a two-to-seven fold increased risk of abortion (Thurmond & Hietala 1997; Paré et al., 1997; Moen et al., 1998; Wouda et al., 1998; Davison et al., 1999; Jensen et al., 1999; Corbellini et al., 2002). Aborting cows usually do not show any other signs of illness. *N. caninum* seropositive cows may be more likely to be prematurely culled (Thurmond & Hietala., 1996) and have a lower milk production than seronegative cohorts (Thurmond & Hietala 1997). However, *N. caninum* has not been found to be a cause of early embryonic death (Björkman et al., 1996; Jensen et al., 1999).

Most congenitally infected calves are also clinically normal, although central nervous signs varying from ataxia to tetraparesis have been reported (Barr et al., 1993). Whilst one study found an association between *N. caninum* seropositivity and decreased post-weaning weight gain in male beef calves (Barling et al., 2001), another study found an increased survival rate in congenitally infected female calves up to 90 days of age in comparison to uninfected calves (Paré et al., 1996). There is therefore no convincing evidence that the parasite causes significant problems in the non-pregnant animal.

**Endogenous transplacental transmission**

Endogenous transplacental spread, resulting from the recrudescence of bradyzoite cysts in the brain, is the principle natural route of transmission of *N. caninum* in cattle. This transmission is highly efficient, with studies suggesting than 81 – 100% of calves born to infected dams are infected (Paré et al., 1996;

Anderson et al., 1997; Wouda et al., 1998; Davison et al., 1999). This is in contrast with *T. gondii* infection, when repeat congenital transmission in successive pregnancies has not been observed (Innes, 1997). Also seropositive cows are more likely than seronegative cows to abort (Thurmond et al., 1997; Moen et al., 1998; Wouda et al., 1998; Davison et al., 1999). This suggests reactivation of an established persistent infection, possibly triggered by the "down regulation" of cell-mediated immunity that occurs around mid-gestation (Innes et al., 2001; Innes et al., 2002; Innes et al., 2005). This is in agreement with human toxoplasmosis, where there is evidence that if host immunity is modified, a persistent infection may become reactivated and cause acute clinical illness (Wreghitt & Joynson, 2001). However, although *N. caninum* infection is common, and transplacental spread of the parasite is highly efficient, only a small proportion of infected cattle abort (Trees et al., 1999).

**Exogenous transplacental transmission**

Despite the efficiency of endogenous transplacental transmission, mathematical models have demonstrated that post-natal infection is still required to maintain *N. caninum* infection within the cattle population (French et al., 1999). A primary infection results from oral ingestion of sporulated oocysts (de Marez et al., 1999; Gondim et al., 2002; Trees et al., 2002, McCann et al., 2007). It is likely that the oocysts excyst in the small intestine, each releasing eight sporozoites, as in ovine toxoplasmosis (Buxton, 1998). It is hypothesised that the sporozoites then parasitise the intestinal epithelium, transform into tachyzoites and undergo a phase of multiplication, possibly in the mesenteric lymph nodes. From here, tachyzoites are released into the blood but it is not known to what extent they are

intracellular or cell-free. *N. caninum* DNA has been demonstrated in blood leucocytes from naturally infected cattle (Okeoma et al., 2005). Lactogenic transmission has been demonstrated experimentally (Uggla et al., 1998; Davison et al., 2001), but adult cows failed to become infected with *N. caninum* when fed homogenised placenta from cows that had given birth to infected calves (Davison et al., 2001).

Prospective studies have demonstrated that horizontal transmission rates are low, with 2/100 heifer years at risk (Davison et al., 1999b), or a rate of less than 1% per year (Hietala & Thurmond, 1999). However, there is evidence that point-source outbreaks can occur. In a study of eight Dutch dairy herds the majority of seropositive cows in a high seropositive age group had seronegative dams or seronegative daughters, in contrast to the rest of the herd where there was a strong association between the serostatus of dam-daughter pairs (Dijkstra et al., 2001b).

**Why do some cattle abort?**

It is not known why some *N. caninum* infected cattle abort and what causes death of the foetus. The processes altering this normally harmless infection to produce fatal consequences are unclear. Experimental studies have shown that the time-point of parasitaemia during pregnancy is critical in determining foetal survival. Foetuses of cows infected intravenously with tachyzoites at 70 days gestation died three to five weeks post infection. Cows infected at 210 days gestation had pregnancies that continued to full term and gave birth to live, persistently infected calves (Williams et al., 2000). The importance of stage of gestation was confirmed in a further experiment, which demonstrated that the timing of parasite

recrudescence in persistently infected pregnant cows also determined foetal survival (Guy et al., 2001). Recrudescence was monitored by an antibody-detection ELISA. In one animal recrudescence occurred at 115 days of gestation and the foetus aborted. In the remaining animals, recrudescence was detected between 168-266 days of gestation, and calves were born persistently infected. When no recrudescence occurred, calves were born uninfected (Guy et al., 2001). It is clear that the timing of parasitaemia, either with *de novo* infection or parasite recrudescence in chronically infected cattle is crucial in determining the outcome of infection. It is possible that the stage of pregnancy could alter maternal immunity to the parasite, or could be due to the stage of foetal development at the time of parasitaemia. Other factors to consider are the magnitude of the parasitaemia and parasite strain virulence (reviewed in Buxton et al., 2002a).

In pregnancy, complex immunological mechanisms exist to allow a dam to nurture a foetus and not reject it as foreign, since the foetus expresses both maternal and paternal MHC antigens. Cytokines that are beneficial to the maintenance of pregnancy (IL-10 and TGF-β) predominate, whereas cytokines potentially detrimental to pregnancy (IL-2, IL-12, IFN-γ and TNF-α) are restricted (Entrican, 2002). This immunological balance during pregnancy may leave the animal more susceptible to infections normally controlled by type 1 helper cell driven, cell mediated responses, including *N. caninum* infection. Invasion of the placenta with *Neospora* tachyzoites could stimulate an immune reaction that is detrimental to pregnancy. It has been shown in humans that IFN-γ and TNF-α can inhibit embryonic development (Haimovici et al., 1991). In sheep, it has been suggested that TNF-α produced by the foetus in response to

*Chlamydophila abortus* infection could play a significant role in abortion (Buxton et al., 2002b). It is possible that maternal and/or foetal Th1 responses, stimulated during pregnancy as a result of infection, could be detrimental to the maintenance of pregnancy.

Alternatively, foetal death could be the direct result of parasite multiplication. The foetal immune system develops throughout gestation (Osburn, 1986), therefore only the older foetus may be capable of controlling parasitaemia and surviving. Foetal immunocompetence starts to develop at 100 days gestation, but only after 150 days gestation is the foetus able to recognise and respond to antigens (Osburn, 1986). This suggests that the foetus would be more likely to survive if infected later in gestation, which is consistent with recent studies (Williams et al., 2000; Guy et al., 2001). Previous findings support the hypothesis that foetal death is a direct result of parasite multiplication and associated pathological lesions, as more protozoa were identified in foetuses less than 5 months old (Otter et al., 1995), whereas older foetuses showed a greater inflammatory response associated with the parasites (Barr et al., 1991).

**Pathological changes in the bovine placenta associated with neosporosis**

In bovine neosporosis, descriptions of placental lesions are limited. Focal necrosis and placentitis have been recorded both in field cases (Barr et al., 1991), and experimental cases (Barr et al., 1994, Macaldowie et al., 1994).

Experimental infections have shown that when *N. caninum* invades cells in the bovine uterus, it causes focal destruction at the maternofoetal interface by

multiplying in maternal and foetal tissue and elicits a largely non-suppurative inflammatory response (Maley et al., 2003; Macaldowie et al., 2004). In cows inoculated with tachyzoites at 70 days gestation within 14 days parasite multiplication within foetal placental villi was evident with villous necrosis, serum leakage between foetal villus and maternal septum, and non-suppurative inflammation in the maternal septa (Macaldowie et al., 2004). Infiltrating maternal inflammatory cells were represented by CD4+, CD8+ and γδ T-cells, and in-situ hybridization showed a proportion of the cells in the infiltrate to produce IFN-γ (Innes et al., 2005).

Reports of naturally occurring infections also describe a non-suppurative placentitis, which in some cases extends into the intercotyledonary chorioallantois (Otter et al., 1995) and varying degrees of mineralization of the villous connective tissue (Barr et al., 1991; Otter et al., 1995; Bergeron et al., 2001).

**Pathological changes in the bovine foetus associated with neosporosis**

Following placental infection, the parasite can invade the foetus, and multifocal non-suppurative encephalitis and myocarditis are the most frequently recorded lesions (Wouda, 2000). If severe, it is possible that these lesions would be sufficient to cause foetal death (Dubey and Schares, 2006).

It is considered that the parasite has a predilection for the central nervous system (CNS) and is initially located in and around blood vessels (Barr et al., 1991; Dubey et al., 1992). In the younger foetus, uncontrolled parasite multiplication can cause lethal widespread destruction of the neuropil, with little or no

inflammation (Ogino et al., 1992; Buxton et al., 2002). In older foetuses, multiplication is more restricted, and necrosis is confined to small foci surrounded by a relatively intense foetal inflammatory infiltrate containing microglia, reactive astrocytes, macrophages and lymphocytes (Barr et al., 1994; Otter et al., 1995; Wouda et al., 1997; Schock, et al., 2000), and these foci may become mineralized (Boulton et al., 1995; Gonzáles et al., 1999). Associated mild meningitis may also be present. Destruction of foetal cells and associated inflammation has been reported in several tissues in association with parasites including the heart, skeletal muscle, lung and liver (Anderson et al., 1991; Barr et al., 1991; Wouda et al., 1997).

**Diagnosis of neosporosis in cattle**

Definitive diagnosis of *Neospora*-associated abortion is challenging, since infection is common and not all infected animals abort. Diagnosis is usually based on the interpretation of several investigations including maternal and foetal serology, histopathology, immunohistology and molecular biological techniques.

The presence of specific antibodies in serum from an aborted cow indicates exposure to *N. caninum* only. Serological tests that can be used to detect *N. caninum* antibodies include ELISA, an indirect fluorescent antibody test (IFAT), and a direct agglutination test (Conrad et al., 1993; Björkman et al., 1994; Paré et al., 1995; Baszler et al., 1996; Björkman et al., 1997; Williams et al., 1997). Serology of the dam reveals high antibody levels in the two weeks following abortion in most cases (Wouda et al., 1998). Therefore, serology performed at the time of abortion can be a valuable tool. It has also been demonstrated that

maternal serology and foetal histology correlate well when used as diagnostic indicators (Otter et al., 1997). An IgG avidity ELISA has been developed for detecting cattle recently infected with *N. caninum*, such that a low avidity index suggests recent *N. caninum* infection (Bjorkman et al., 1999).

Foetal serology, using serum or fluid from body cavities, can be performed on foetuses over 5 months, when the foetus starts to produce antibodies (Dubey and Schares, 2006). Therefore this technique is not useful in foetuses below this age. Additionally, congenitally infected calves will also produce antibodies so this does not prove that abortion was *Neospora*-associated (Paré et al., 1996).

Examination of foetal tissues is considered necessary for a definitive diagnosis of neosporosis. There are no pathognomonic gross lesions, but histological examination has a high sensitivity and specificity (Otter et al., 1997). Histological lesions characteristic of *Neospora* infection are multifocal non-suppurative encephalitis and myocarditis (Wouda, 2000).

Definitive confirmation of infection depends on the identification of parasites in tissues. Immunohistology (IH) is applied for this purpose, using polyclonal antiserum from rabbits immunised with cell culture-derived tachyzoites. (Lindsay & Dubey, 1989). However, polyclonal *N. caninum* antibodies sometimes cross-react with *T. gondii* (Dubey & Lindsay, 1996). Several *N. caninum*-specific monoclonal antibodies have also been developed, but as yet no antibody is commercially available for IH use. The main advantage of this technique is that the

presence of parasites can be observed in relation to histological lesions (Wouda, 2000).

PCR and in situ hybridisation techniques have been developed for the detection of *Neospora* DNA (De Marez et al., 1999; Holmdahl & Mattsson, 1996; Jenkins et al., 2002; Uggla et al., 1998). These techniques generally have a high sensitivity and specificity for the detection of parasites (Wouda, 2000).

**Neosporosis in relation to other abortifacients in ruminants**

In toxoplasmosis in sheep, it is considered that foetal death results from anoxia due to placental damage caused by the parasite (Buxton et al., 1982). Although a closely related parasite, it has not as yet been demonstrated that placental damage caused by *N. caninum* does indeed cause abortion. Placentitis is considered to be the cause of abortion with several abortifacients in cattle including *Bacillus licheniformis* and *Listeria monocytogenes* (Johnson et al., 1994). In other abortifacients, such as *Salmonella enterica* serovars, abortion is considered to be due to the systemic illness that it causes in the dam (Radke et al., 2002).

The immunocompetence of the foetus has important implications in bovine virus diarrhoea virus (BVDV) infections. Before 100 days gestation, the bovine foetus is unable to recognise BVDV as being foreign (reviewed in Nettleton and Entrican, 1995) and calves that survive infection at this stage are born immunotolerant to the virus, being persistently infected but seronegative. At 100–150 days gestation, the foetus starts to be able to mount an immune response (Osburn, 1986) and after 150 days, it becomes progressively more competent at recognizing and responding

in full to BVDV. Abortion in the case of BVDV infection occurs most commonly before the foetus is fully immunocompetent (Brownlie et al., 1987).

**Control measures for neosporosis in cattle**

Currently, there are no available drug therapies or vaccines in the UK for the control of bovine neosporosis. Decoquinate has been demonstrated to kill intracellular *N. caninum* tachyzoites *in vitro* at 0.1ug/ml, but the effect of the drug *in vivo* is unknown (Lindsay et al., 1997). Toltrazuril and ponazuril medication at 20 mg/kg/d was effective at preventing detectable parasites in the brains of mice and exogenously challenged calves (Gottstein et al., 2001; Kritzner et al., 2002). A vaccine is available in the USA (Neoguard, Intervet); however its efficacy against preventing in-utero transmission and abortions due to *de novo* challenge are not fully understood (Andrianarivo et al., 2000, Choromanski & Block, 2000), although a recent field trial in Costa Rica demonstrated that vaccination was associated with a 46% decrease in abortion rate (Romero et al., 2004). Cattle infected with *N. caninum* prior to mating were protected against vertical transmission of the parasite following a further challenge in mid-gestation (Innes et al., 2001).

Williams et al. (2003) demonstrated that immunity to a pre-existing infection in chronically infected cattle protected against an exogenous challenge at 70 days gestation, but that this protective immunity did not prevent transplacental infection of the pre-existing infection. Additionally, it was found that infection with live tachyzoites prior to pregnancy protected against foetal death following a

further experimental infection at 70 days gestation (Williams et al., 2007). These observations suggest that vaccination as a means of control against exogenous transplacental transmission of neosporosis is a future prospect. However, preventing abortions resulting from endogenous infection through vaccination will be a challenge (Williams and Trees, 2006).

In the absence of an effective drug or vaccine, and since endogenous transplacental transmission has been identified as a major factor contributing to the persistence of infection, culling infected animals has been advocated (Thurmond & Hietala, 1997). Since the prevalence of infection is high on many infected farms, this is often impractical. More commonplace is the exclusion of calves from infected dams as replacement stock (Thurmond & Hietala, 1995). In a deterministic mathematical model both of these methods were effective in reducing herd seroprevalence levels over several years (French et al., 1999). In the case of valuable breeding stock, embryo transfer into seronegative recipients has been successful (Thurmond & Hietala, 1996).

Finally, control measures against exogenous transplacental transmission are advocated, since only controlling against endogenous transplacental transmission will result in a low herd seroprevalence but will not eradicate the parasite (French et al., 1999; Clancy and French, 2001). Therefore, since the dog has been identified as a definitive host of the parasite, it is advisable to limit the access of dogs to aborted foetuses and foetal membranes, and to prevent the contamination of grazing areas and feed by dog faeces (Wouda, 2000).

**Aims and objectives of the study**

In the last two decades, research into *N. caninum* has resulted in a greater understanding of the epidemiology of neosporosis in cattle. However the fundamental scientific question of how this parasite causes abortion in cattle remains. Addressing this question will enhance our understanding of the pathogenicity of the parasite and potentially lead to more efficient ways to control a disease that has major welfare and economic importance to the livestock industry.

It is proposed that *N. caninum* could be capable of causing abortion by maternal placental inflammation, maternal and foetal placental necrosis or damage to foetal tissues. We hypothesise that the parasite causes death by multiplying uncontrollably in an immature foetus and therefore the aim of this study is to investigate the pathogenesis of the parasite in the placenta and foetus. We will compare the distribution of parasites (by polymerase chain reaction, immunohistology and transmission electron microscopy) and the histopathological changes in the placenta and foetus following experimental infection of cattle with *N. caninum* in early gestation (when foetal death occurs) and late gestation (when the foetus survives). Parasite distribution and histopathological changes will also be investigated in placentae and foetuses following endogenous transplacental transmission in chronically infected cattle.

# Chapter two

## Comparison of the sensitivity of two different PCR methods for the detection of *Neospora caninum* DNA.

## 2.1 Abstract

The polymerase chain reaction (PCR) is a commonly used tool for the molecular diagnosis of parasitic infections and several PCR protocols have been developed for the detection of *N. caninum* DNA in tissues. The aim of this work was to determine the relative diagnostic sensitivity of two PCR protocols routinely used within our laboratory for the detection of *N. caninum* in tissues from aborted foetuses. Method one was a nested PCR method based on the internal transcribed spacer 1 region of the ribosomal RNA sequence and method two was a single step PCR designed to amplify a fragment of Nc5 *N. caninum*-specific genomic DNA. A system was devised to extract and amplify *N. caninum* DNA in brain tissue seeded with serial dilutions of known numbers of tachyzoites. The two protocols were carried out in parallel and their sensitivities compared.

Method one consistently detected *N. caninum* specific DNA from brain samples seeded with 10 or more tachyzoites. In contrast, method two had a limit of detection of *N. caninum* specific DNA from brain samples seeded with $10^5$ or more tachyzoites. In conclusion, in the environment of our laboratory, method one is the more sensitive of the two PCR protocols investigated.

## 2.2 Introduction

The last three decades have seen the emergence of an important parasite in cattle, *Neospora caninum*. The development of diagnostic tools has been a major element of the research carried out since the parasite was first described. The first identification was of a protozoan parasite, distinct from *Toxoplasma gondii* in Norwegian dogs causing severe encephalitis (Bjerkas et al., 1984). This parasite was subsequently characterised and classified by Dubey and co-workers in 1988 (Dubey et al., 1988a). The clinical syndrome described in dogs by Bjerkas et al. (1984) was not observed in other species with toxoplasmosis and antibodies to *T. gondii* were not present in these dogs. Finally, tissue cysts of this parasite were morphologically distinct. Therefore, a new genus, Neospora was proposed to accommodate this species, *N. caninum*, which was considered not to fit into existing genera. After this description, the parasite was isolated in cell culture, inoculated into mice, and the subsequent finding of thick walled tissue cysts confirmed the new species (Dubey et al., 1988b). This new parasite quickly emerged as an important cause of abortion in cattle worldwide (Dubey and Lindsay, 1996).

With the emergence of this important parasite came the challenge of developing accurate and reliable diagnostic tests. This challenge was complicated by the biology of the parasite. Its transmission to the foetus in pregnant cattle can result in abortion, or the birth of calves that are persistently infected. This transplacental spread, resulting from the recrudescence of bradyzoite cysts in the brain, is the principal natural route of transmission of *N. caninum* in cattle.

Transmission is highly efficient, with 95% of calves born to infected dams being infected (Davison et al., 1999). However, although infection is common, and transplacental spread of the parasite is highly efficient, only a proportion of infected cattle abort (Trees et al., 1999).

Shortly after the description of the parasite, a serological test (Dubey et al., 1988b) and an immunohistological method (Lindsay and Dubey, 1989) were developed to diagnose infection. Several researchers described the histopathology of aborted foetuses and the most common lesions were multifocal non-suppurative encephalitis and myocarditis (Barr et al., 1991; Lindsay et al., 1993; Wouda et al., 1997). Serology, histopathology and immunohistology remain the principal techniques used to diagnose *Neospora*–associated abortion (Dubey and Lindsay, 1996). More details of the methods employed to diagnose *Neospora*–associated abortion are given elsewhere in this thesis.

There has been increasing interest in studying *N. caninum* at the molecular level, aiming both to characterise the genome and develop diagnostic PCR tests. These tests have been developed using specific oligonucleotide sequences (primers) to identify and amplify regions of the *N. caninum* genome. Progress in the development of PCR protocols for *N. caninum* has been rapid, due to prior work on *T. gondii,* as *N. caninum* and *T. gondii* are closely related at the molecular level (Ellis, 1998).

Several PCR methods were developed for the detection of *T. gondii* using the ribosomal DNA unit, since it is present in a high copy number, and this was an

obvious choice for development of the first PCR tests for *N. caninum*. Differences between the parasites are detectable in the sequences of the ITS1 region of the ribosomal RNA gene. This region is useful for PCR, since it shows variability between species but is conserved within species, and is present in a high copy number (Holmdahl & Mattsson, 1996). Using the ITS1 region, Holmdahl & Mattsson (1996) designed *N. caninum* specific primers. This method was modified by Uggla et al. (1998), who developed a nested PCR, using external primers complementary to regions of the rDNA unit, then internal *N. caninum* specific primers complementary to ITS1.

Several other PCR protocols have been developed based on rDNA. The ITS1 region was also the target of a PCR developed by Payne and Ellis (1996). Ellis et al. (1999) later modified this approach to a single tube nested PCR for increased sensitivity. Ho et al. (1996) described an alternative PCR approach using universal primers to amplify 18S rDNA. *N. caninum* PCR products were then identified by species-specific chemiluminescent PCR probes.

In 1996, Muller et al. designed *N. caninum* specific primers (Np21plus and Np6plus) to amplify a region of genomic DNA for use in PCR. This 337nt long fragment of Nc5 *N. caninum*-specific genomic DNA is an anonymous piece of DNA, which is not expressed and has no known function (Kaufmann et al., 1996). In 1999, De Marez et al. modified this PCR by increasing the number of amplification cycles to 40 for a higher level of sensitivity.

To date, no direct comparison of the sensitivity of these commonly used PCR diagnostic techniques has been made in our laboratory. However, in most cases some indication of the sensitivity of each method was given. Using the ITS1

region, Holmdahl & Mattsson (1996) designed *N. caninum* specific primers that were found to detect five or more tachyzoites from cell culture. Uggla et al. (1998) did not report the sensitivity when this method was modified into a nested PCR.

Sensitivity was not assessed with the PCR developed by Payne and Ellis (1996), again targeting the ITS1 region. The single tube nested PCR method of Ellis et al. (1999) detected a product from dog brain seeded with 10fg of genomic DNA. Ho et al. (1996), amplifying 18S rDNA, described a sensitivity of five or more tachyzoites in 100µl bovine whole blood or amniotic fluid.

The PCR method of Muller et al. (1996), amplifying an anonymous region of genomic DNA, was sensitive to a single tachyzoite in the absence of other tissue, 10 tachyzoites in skeletal muscle or 100 tachyzoites in brain tissue. When De Marez et al. (1999) modified this PCR for a higher level of sensitivity, the increased sensitivity was not assessed.

For the diagnosis of *N. caninum* in infected tissues, the sensitivity of these methods to detect parasites in specific tissues needs to be addressed. The aim of this study was to compare the sensitivity of two PCR methods at detecting known numbers of parasites in portions of bovine brain tissue, and to ensure reproducibility of the results.

## 2.3 Materials and methods

### 2.3.1 Experimental design

To date, both the PCR method of Uggla et al. (1998) and that of De Marez et al. (1999) have been regularly used in our laboratory for the detection of *N. caninum* in tissues. The aim of this work was to determine which of these protocols was more sensitive at detecting parasites in infected bovine brain tissue. In order to determine the relative sensitivity of these two PCR protocols, a method was devised to extract and amplify *N. caninum* DNA from tissues containing known numbers of tachyzoites. Portions of brain tissue from a calf born from a serologically negative dam and with no evidence of pre-colostral *N. caninum* antibodies were seeded with dilutions of *N. caninum* Liverpool strain tachyzoites grown and purified from cell culture. The numbers of tachyzoites seeded were log dilutions from $10^6$ to 1. DNA was then extracted from these seeded tissues. DNA was also extracted from unseeded brain tissue and from $10^6$ tachyzoites for use as negative and positive controls respectively. The PCR protocol of Uggla et al. (1998) was termed method one and that of De Marez et al. (1999) was termed method two. Methods one and two were carried out on the extracted DNA in parallel on two separate occasions. The entire process of seeding brain with cultured tachyzoites, DNA extraction, and the parallel running of both protocols was then repeated to ensure that the results were reproducible.

### 2.3.2 Preparation of samples and DNA extraction

*2.3.2.1 Culture of tachyzoites*

Vero cell cultures were established using methods described previously (Williams et al., 2000). 80 $cm^2$ flasks were inoculated with $1 \times 10^5$ cells in 5 ml RPMI 1640 medium supplemented with 2 % horse serum (Invitrogen, Paisley, UK) and penicillin/streptomycin (100 µg/ml; BioWhittaker, Walkersville, USA). Cultures were incubated at 37 °C with 5 % $CO_2$. When cells were confluent, they were infected with a parasite infected cell culture at $1 \times 10^5$ parasites per flask. After 24 h, the media was replaced with RPMI 1640 medium supplemented with penicillin/streptomycin only. Parasites were harvested when free tachyzoites were visible in the culture. Cells were dislodged with a cell scraper and centrifuged at 1000 g for 10 min. The cell pellet was re-suspended in 10 ml sterile phosphate buffered saline (PBS, pH 7.2) and centrifuged at 1000 g for 10 min. The cells were then suspended in 2.5 ml of sterile PBS and disrupted by passing through decreasing gauge blunt ended needles. The sample was then passed through PD10 columns pre-packed with Sephadex G25 medium (Pharmacia, Uppsala, Sweden), which allows passage of parasites only. The number of tachyzoites collected was calculated using a Fuchs Rosenthal counting chamber.

*2.3.2.2 Preparation of DNA*

Brain from a newborn calf serologically negative for *N. caninum* was seeded with *N. caninum* tachyzoites. The harvested tachyzoites were aliquoted into 100 µl volumes containing log dilutions of tachyzoites from $10^6$ to 1. Each aliquot was added to a 25 mg sample of brain. DNA was extracted using the DNeasy® tissue extraction kit (Qiagen Ltd, Crawley, UK), following the manufacturer's

instructions for animal tissues. This kit uses advanced silica-gel-membrane technology for rapid purification of total cellular DNA. Samples were lysed with proteinase K and buffering conditions were adjusted to provide optimal binding conditions before the lysate was loaded onto the DNeasy® mini spin column. During centrifugation, DNA was selectively bound to the DNeasy® membrane. Contaminants and enzyme inhibitors were removed by two wash steps, and DNA was eluted into 100 µl of buffer, ready for use. The same method was used to extract DNA from $10^6$ tachyzoites in 100 µl PBS for use as a positive control and from 25 mg of brain from the same newborn calf serologically negative for *N. caninum* for use as a negative control.

### 2.3.3 PCR method one

*2.3.3.1 Reagent concentrations for PCR method one*

In the first PCR the samples comprised 3 µl, and 2 µl in the second PCR. The samples for the second reaction were handled in a room separate from the first reaction in order to minimise the risk of contamination. The 50 µl reaction mixtures used for each step contained the sample, 200 µM of each deoxynucleotide, Hot star Taq (Qiagen Ltd, Paisley, UK, 2.5 units per reaction), and Hot star Taq buffer with a final concentration of 1.5 mM MgCl. Primer concentrations were 0.1 µM and 0.4 µM in reactions 1 and 2, respectively. Cultured *N. caninum* tachyzoite DNA and ultrapure water were used as positive and negative controls respectively.

*2.3.3.2 Oligonucleotide primer sequences for PCR method one*

The external primers, F6 and 5.8B, 5′- CAG GTC TGT GAT GCC C-3′ and 5′-TCG CGT TTT GCT GCG TTC TTC-3′, respectively, were complementary to regions of the rDNA unit (18S and 5.8S), and used in the first PCR. The internal primers PN3 and PN4, 5′-TAC TAC TCC CTG TGA GTT G-3′ and 5′-TCT CTT CCC TCA AAC GCT A-3′, respectively, specific for *N. caninum*, were complementary to ITS1, and were used in the second PCR. Primers were prepared by Sigma Genosys, Dorset, UK.

*2.3.3.3 Reaction conditions for PCR method one*

Initially the samples were heated for 2.5 min at 94 °C; thereafter each cycle consisted of a 30 sec denaturation at 94 °C, 40 sec at the annealing temperature of 50 °C, followed by a 1 min extension at 72 °C. Amplification was performed with F6 and 5.8B over 25 cycles followed by a final extension at 72 °C for 3 min. In the second PCR the annealing temperature was increased to 54 °C, extension decreased to 30 sec and the amplification was performed with PN3 and PN4 over 30 cycles, as above.

**2.3.4 PCR method two**

*2.3.4.1 Reagent concentrations for PCR method two*

In this PCR protocol the samples comprised 2 µl. The 50 µl reaction mixtures used for each step contained the sample, 200 µM of each deoxynucleotide, Hot star Taq (Qiagen Ltd, Paisley, UK, 2.5 units per reaction), and Hot star Taq buffer with a final concentration of 1.5 mM MgCl. The primer concentration was 0.2 µM. Cultured *N. caninum* tachyzoite DNA and ultrapure water were used as positive and negative controls respectively.

*2.3.4.2 Oligonucleotide primer sequences for PCR method two*

The primers, NP21+ and NP6+, 5'- CCC AGT GCG TCC AAT CCT GTA AC-3' and 5'-CTC GCC AGT CAA CCT ACG T CT TCT-3', respectively, were complementary to a 337nt long fragment of Nc5 *N. caninum*-specific genomic DNA. Primers were prepared by Sigma Genosys, Dorset, UK.

*2.3.4.3 Reaction conditions for PCR method two*

Initially the samples were heated for 1.5 min at 95 °C; thereafter each cycle consisted of a 30 sec denaturation at 94 °C, 40 sec at the annealing temperature of 64 °C, followed by a 1 min extension at 72 °C. Amplification was performed over 40 cycles followed by a final extension at 72 °C for 3 min.

**2.3.5 Agarose gel electrophoresis**

10 µl aliquots of reaction mixtures were analysed by 2 % agarose gel electrophoresis and the products stained with SYBR safe® DNA gel stain (Invitrogen, Paisley, UK) and visualised under U.V. light.

## 2.4 Results

### 2.4.1 PCR method one detects *N. caninum* DNA in all brain samples seeded with 10 or more tachyzoites

This protocol was the nested PCR method of Uggla et al. (1998). To determine the sensitivity of this PCR protocol, it was used to amplify *N. caninum* DNA from tissues containing known numbers of tachyzoites. DNA extracted from $10^6$ tachyzoites was the positive control. A single 249kb amplification product was detected, which was consistent with the published fragment length using these primers (Fig. 2.4.1, lane 9). No products were detected from the uninfected brain sample (Fig. 2.4.1, lane 8) or the water (Fig. 2.4.1, lane 10). In the samples of brain seeded with log dilutions of tachyzoites, *N. caninum* specific DNA was detected in all brain samples seeded with 10 or more tachyzoites (Fig. 2.4.1, lanes 1-6). In the brain sample seeded with one tachyzoite, no amplification product was detected (Fig. 2.4.1, lane 7). The PCR was repeated with the same extracted DNA with the same result. The whole process of seeding samples with tachyzoites, extracting DNA and performing the PCR reaction was repeated again with the same result.

### 2.4.2 PCR method two detects *N. caninum* DNA in all brain samples seeded with $10^5$ or more tachyzoites

This protocol was the PCR method of De Marez et al. (1999). To determine the sensitivity of this PCR protocol, it was used to amplify *N. caninum* DNA from the same samples used for PCR method one. The extracted DNA from $10^6$

tachyzoites was the positive control and a single 337kb amplification product was detected, which was consistent with the published fragment length using these primers (Fig. 2.4.2, lane 9). No products were detected from the uninfected brain sample (Fig. 2.4.2, lane 8) or the water (Fig. 2.4.2, lane 10). In the samples of brain seeded with log dilutions of tachyzoites, this method detected *N. caninum* specific DNA from brain samples seeded with $10^6$ and $10^5$ tachyzoites (Fig. 2.4.2, lanes 1 and 2). In the brain samples seeded with $10^4$ or less tachyzoites, no amplification products were detected (Fig. 2.4.2, lanes 3 - 7). The PCR was repeated with the same extracted DNA with the same result. The whole process of seeding samples with tachyzoites, extracting DNA and performing the PCR reaction was repeated again with the same result.

**Figure 2.4.1: Agarose gel (2%) electrophoresis of the products obtained by PCR method one (Uggla et al., 1998).**

Lanes 1-7 – calf brain seeded with $10^6$ ( lane 1), $10^5$ (lane 2), $10^4$ (lane 3), $10^3$ (lane 4), $10^2$ (lane 5), 10 (lane 6) and 1 (lane 7) tachyzoites. Lane 8 – uninfected cow brain. Lane 9 – positive control, tachyzoite DNA. Lane 10 – negative control, water. A 100-bp ladder (M) from Bioline Limited (London, UK) was used as a molecular size marker.

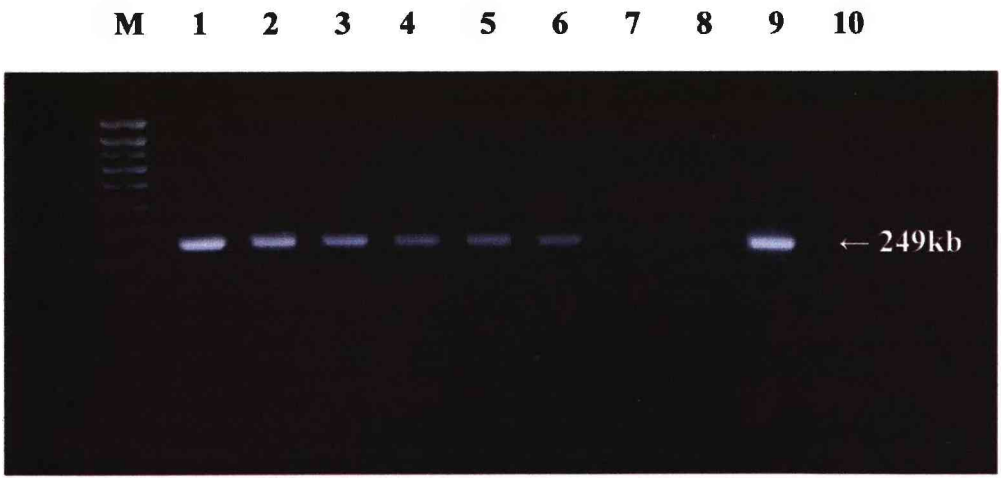


**Figure 2.4.2: Agarose gel (2%) electrophoresis of the products obtained by PCR using method two (De Marez et al., 1999).**

Lanes 1-7 – calf brain seeded with $10^6$ ( lane 1), $10^5$ (lane 2), $10^4$ (lane 3), $10^3$ (lane 4), $10^2$ (lane 5), 10 (lane 6) and 1 (lane 7) tachyzoites. Lane 8 – uninfected calf brain. Lane 9 – positive control, tachyzoite DNA. Lane 10 – negative control, water. A 100-bp ladder (M) from Bioline Limited was used as a molecular size marker.

## 2.5 Discussion

PCR has been widely used for the detection of *N. caninum* DNA, and several different methods have been described (Ellis, 1998). However, the sensitivities of such approaches at detecting parasites in infected tissues have not been fully described. This study investigated the relative sensitivities of two of the most commonly used PCR protocols for the diagnosis of *N. caninum* infection in bovine tissues. Furthermore, the level of sensitivity observed for each method was reproducible since consistent results were obtained on three separate occasions.

PCR method one consistently detected *N. caninum* specific DNA from all brain samples seeded with 10 or more tachyzoites. Further investigation of the cut off of sensitivity between 10 and 1 tachyzoite was not performed, so sensitivity could be less than 10 tachyzoites. No published data for the sensitivity of this method is available, however this protocol was modified from the method of Holmdahl and Mattson (1996), which detected *N. caninum* DNA in samples of five or more tachyzoites from cell culture. Therefore, our findings are consistent with the sensitivity of the PCR method from which it is based, and suggest that sensitivity is not lost when DNA is extracted from parasites in tissue rather than directly from cell culture. Although the specificity of this method was not investigated in this experiment, previous findings have suggested that reactions based on the ITS-1 sequence are specific, since it is conserved within species but variable between species (Holmdahl and Mattson, 1996; Payne and Ellis, 1996).

PCR method two consistently detected *N. caninum* specific DNA from all brain samples seeded with $10^5$ or more tachyzoites. Again, further investigation of the cut off of sensitivity between $10^5$ and $10^4$ tachyzoites was not performed. This is markedly different from the published sensitivity for the method of Muller et al. (1996), which detected DNA extracted from 100 tachyzoites in brain tissue. However, demonstration by gel electrophoresis of amplification products using this PCR was often substantially hampered by the existence of background smears at the predicted location of the diagnostic band. In addition De Marez et al. (1999), who used this PCR, but increased the number of amplification cycles to 40, reported that DNA was not always detected in every repeat reaction with the same sample. These observations raise questions over the reliability and repeatability of this method. Again specificity of this method was not investigated in this experiment, but Muller et al. (1996) tested DNA amplified from *Hammondia hammondi, Sarcocystis* spp. and *T. gondii*, and no products were found.

In summary, in the environment of our laboratory, method one is the more sensitive of the two PCR protocols investigated. When considering why method one was the more sensitive, several factors should be considered. Firstly, the fact that method one was the nested PCR method and therefore consisted of two rounds of DNA amplification is one explanation for increased sensitivity. Secondly, the fact that method one targets a highly repeatable and conserved region, whereas method two targets a region of genomic DNA of no known function could be another reason for the increased sensitivity of method one, owing to the potential for variation of the region of genomic DNA. Thirdly,

method one starts with a larger quantity of DNA than method two, which may increase sensitivity. Finally, the repeatability and reliability problems of method two outlined earlier could be a factor in the reduced sensitivity of this method.

One problem with both of these PCR methods for the detection of *N. caninum* DNA in tissues will always be that no conclusions can be drawn about differences in parasite intensity between samples. Although different band intensities can suggest different quantities of parasite DNA, the only certainty is whether parasite DNA is present or not in that sample. In the last few years, methods to quantify parasite DNA using real-time PCR have been published. Dual hybridization probes and a LightCycler™ machine were used for quantitative assessment of *N. caninum* proliferation in organotypic slice cultures of tissue from the rat central nervous system (Muller et al, 2002). *N. caninum* DNA in the blood and brains of pregnant and aborting heifers was quantified by monitoring PCR product formation in real-time using SYBR Green 1 and primers designed to amplify a 180bp product specific to *N. caninum* from the Nc-5 gene fragment (Okeoma et al., 2005). This recent development towards quantifying DNA and therefore being able to find differences between tissues would provide useful additional information. Collantes-Fernández et al. (2006) investigated parasite distribution in naturally aborted foetuses at different stages of gestation using primer pairs for the *N. caninum* Nc-5 sequence together with a real-time PCR to determine parasite loads. This gave important additional information highlighting increased parasite numbers in brain and heart tissues in early gestation compared to foetuses aborted later in gestation.

Unfortunately the technology for quantifying DNA was not available at the time that the experimental work for this thesis was carried out. Had quantification of DNA been possible, it would undoubtedly have enhanced the work.

Another point to note is that the DNA extraction method may play an important role in the diagnostic performance of the tests. Evidence for this is given in the interlaboratory comparison of PCR methods carried out by van Maanen et al. (2004) when no clear relationship was found between laboratories using the same PCR format and the diagnostic sensitivities found. Therefore it should be stressed that the relative sensitivities described for these two methods are only relevant with the DNA extraction method used in this case.

# Chapter 3

## Parasite distribution and lesions after experimental infection of cattle with *Neospora caninum* in early and late gestation.

### 3.1 Abstract

The protozoan parasite *Neospora caninum* is the most frequently diagnosed abortifacient in the UK and a cause of abortion worldwide. It is not fully understood why some infected cattle abort, but previous studies have shown that abortion is more likely if transplacental spread of the parasite occurs early in gestation. Bovine foetal immunocompetence develops gradually during the second half of gestation, and it has been suggested that the immunocompetence of the foetus at the time of infection may determine its ability to control parasitaemia and to survive. To test this hypothesis we have compared the distribution of parasites (by polymerase chain reaction, immunohistology and transmission electron microscopy) and the histopathological changes in the placenta and foetus following experimental infection of cattle with *N. caninum* in early and late gestation. In early gestation, following foetal death, *N. caninum* DNA was detected and evidence of widespread parasite infiltration was demonstrated immunohistologically in the placenta. This was associated with extensive focal epithelial necrosis, serum leakage and a moderate maternal CD4+ T cell-dominated mononuclear cell interstitial inflammatory response. Widespread parasite infiltration was also evident in the foetus, with parasites in most tissues, often associated with necrosis. In late gestation, *N. caninum* DNA was detected sporadically but parasites were not evident immunohistologically in the placenta. Small foci of necrosis were seen occasionally, with a mild CD4+ and CD8+ T cell-dominated mononuclear cell interstitial inflammatory response. *N. caninum* DNA was sporadically detected in the foetus and parasites were demonstrated immunohistologically in brain and spinal cord only, in association with a non-suppurative inflammatory response. This data is consistent with an uncontrolled

parasitaemia in an immunologically immature foetus, which could, via multiparenchymal necrosis, lead to foetal death. Additionally, the widespread necrosis and inflammation observed in the placenta after infection early in gestation could, through hypoxia and/or malnutrition of the foetus, be a contributing factor to abortion.

### 3.2 Introduction

The apicomplexan parasite *Neospora caninum* is the most frequently diagnosed abortifacient in dairy cattle in the UK and is a cause of abortion worldwide (VLA Surveillance Report 2006; Dubey, 1999). Endogenous transplacental transmission, resulting from recrudescence of bradyzoite cysts in a chronically infected dam is the principal natural route of infection (Dubey & Lindsay, 1996). It has also recently been shown that cattle may abort after exogenous transplacental transmission, following ingestion during pregnancy of oocysts from the faeces of dogs, a definitive host (Gondim et al., 2004; McCann et al., 2008). However, although infection is common, and transplacental spread of the parasite is highly efficient, only a proportion of infected cattle abort (Trees et al., 1999). The processes altering this normally benign infection to produce fatal consequences are unclear. Experimental studies have shown that the time of foetal infection during pregnancy is critical in determining foetal survival. Cattle infected intravenously with tachyzoites at day 70 of gestation had foetuses that died three to five weeks post infection, whereas cattle infected at day 210 of gestation had pregnancies that continued to full term and gave birth to live, persistently infected calves (Williams et al., 2000). This finding was supported by a further study of chronically infected animals, where foetal death occurred in one animal when the parasite recrudesced early in gestation, whereas in the remaining animals where the parasites recrudesced later, the foetuses survived (Guy et al., 2001). It is clear that the timing of parasitaemia, either with *de novo* infection or parasite recrudescence in chronically infected cattle, is crucial in determining the outcome of infection.

Foetal death could be the direct result of parasite multiplication. Foetal immunocompetence starts to develop at 100 days gestation, but only after 150 days gestation is the foetus able to recognise and respond competently to antigens (Osburn, 1986), therefore only the older foetus may be capable of controlling parasitaemia and surviving. This suggests that the foetus would be more likely to survive if infected later in gestation, and is consistent with the results of previous studies (Williams et al., 2000; Guy et al., 2001). Previous findings support the hypothesis that foetal death is a direct result of parasite multiplication in an immunologically immature foetus, as more protozoa were identified in foetuses less than 5 months old (Otter et al., 1995). Conversely, some authors have suggested that a foetal immune response to the parasite could in itself be damaging. Older foetuses showed a greater inflammatory response associated with the parasites (Barr et al., 1991). The most frequently observed lesions are multifocal non-suppurative encephalitis and myocarditis (Wouda, 2000) and, if severe, are considered to be sufficient to cause foetal death (Dubey and Schares, 2006).

It is also possible that abortion could occur as a direct result of parasite-induced damage to the placenta. In toxoplasmosis in sheep, it is considered that foetal death results from anoxia due to placental damage caused by the parasite (Buxton et al., 1982). In bovine neosporosis, focal necrosis and placentitis have been recorded both in field cases (Barr et al., 1991) and experimental cases (Barr et al., 1994; Macaldowie et al., 1994).

It is clear that immunological control of the parasite at the level of the placenta or by the foetus, either in a protective or detrimental manner, could be key to

determining the mechanism of abortion. The aim of this study was to investigate the pathogenic processes which take place in the placenta and foetus in association with infection. We have infected cattle with *N. caninum* in early and late gestation and have compared the parasite distribution, pathological lesions and immune cell infiltration in the placenta and foetus at a time where the foetus died with a time where transplacental transmission occurred but the foetus survived.

## 3.3 Materials and methods

### 3.3.1. Project design and sample collection

*3.3.1.1 Source and husbandry of cattle*

Holstein Friesian heifers aged 18-24 months were purchased from a commercial farm. The cattle were tested by the Veterinary Laboratories Agency, Preston, UK, to ensure they had no evidence of exposure to *N. caninum* and were not infected with bovine viral diarrhoea virus. Cattle were treated with Procaine Penicillin and Dihydrostreptomycin Sulphate (Streptacare®, Animalcare Limited, York, UK) at a dose rate of 20 mg/kg penicillin and 25 mg/kg streptomycin by intramuscular injection daily for three days after purchase to treat any chronic leptospiral infections. The cattle were vaccinated against leptospira (Leptavoid-H®, Schering-Plough, Uxbridge, UK), bovine viral diarrhoea virus (Bovidec®, Novartis, Royston, UK) and infectious bovine rhinotracheitis virus (Bovilis IBR marker®, Intervet, Milton Keynes, UK). The cattle were oestrus synchronised using progesterone releasing intra-uterine devices (PRID®, CEVA Animal Health, Watford, UK) and artificially inseminated with certified disease-free Holstein Friesian semen. The cattle were housed with a Hereford bull immediately after insemination. Pregnancy and foetal viability were confirmed at 35 days by trans-rectal ultrasonography. Animals were maintained on straw-bedded pens at Liverpool University Farm, Leahurst Campus with free access to hay and water and fed concentrates twice daily.

*3.3.1.2 Experimental design*

Before the start of the experiments, each animal was allocated randomly to one of four groups of six. Each animal in an infected group received the infective inoculum intravenously (iv) in the jugular vein. This was at day 70 of gestation (70 dg) for each group 1 animal and at day 210 of gestation (210 dg) for each group 3 animal. The control groups (2 and 4) were given an intravenous injection of parasite-free inoculum at the same time. Dams and foetuses were designated numbers as illustrated in table 3.2.1.

**Table 3.3.1 – Experimental Design**

| Cattle Group | Individual animal numbers | Type of inoculation | Timing of inoculation |
|---|---|---|---|
| 1 | I 1 - 6 | $10^7$ *N. caninum* tachyzoites in 2ml PBS | 70dg |
| 2 | C 1 - 6 | Purified uninfected Vero cells in 2ml PBS | 70dg |
| 3 | I 7 - 12 | $10^7$ *N. caninum* tachyzoites in 2ml PBS | 210dg |
| 4 | C 7 - 12 | Purified uninfected Vero cells in 2ml PBS | 210dg |

dg – day of gestation

All cattle were examined by trans-rectal ultrasonography three times a week to monitor foetal viability until day 14 post challenge, and daily thereafter. The cattle were killed after foetal death (day 70 challenge) or 20 to 22 days post challenge in the cases where foetal death did not occur (day 210 challenge).

Animals were killed by captive bolt pistol followed by pithing and dams and foetuses were subjected to a post-mortem examination.

*3.3.1.3 Preparation of inocula*

To prepare the inocula, aliquots of *N. caninum* Liverpool strain (Barber *et al.*, 1997) tachyzoites were removed from liquid nitrogen and thawed. Uninfected Vero cell cultures were established using methods described previously (Williams et al., 2000). 80 $cm^2$ flasks were inoculated with $1 \times 10^5$ Vero cells in 5 ml RPMI 1640 medium supplemented with 2% horse serum (Invitrogen, Paisley, UK) and penicillin/streptomycin (100µg/ml; BioWhittaker, Walkersville, USA). Cultures were incubated at 37 °C with 5% $CO_2$. When cells were confluent, they were infected with a parasite infected cell culture at $1 \times 10^5$ parasites per flask. After 24 h, the media was replaced with RPMI 1640 medium supplemented with penicillin/streptomycin only. Parasites were harvested when free tachyzoites were visible in the culture by light microscopy. Cells were dislodged with a cell scraper and centrifuged at 1000 g for 10 min. The cell pellet was re-suspended in 10 ml sterile phosphate buffered saline (PBS, pH 7.2, see Appendix 1) and centrifuged at 1000 g for 10 min. The cells were then suspended in 2.5 ml of sterile PBS and disrupted by passing through decreasing gauge blunt ended needles. The sample was then passed through PD10 columns pre-packed with Sephadex G10 medium (Pharmacia, Uppsala, Sweden), which allows passage of parasites only. The number of tachyzoites collected was calculated using a Fuchs Rosenthal counting chamber. The number of parasites was adjusted to provide an inoculum of $10^7$ tachyzoites in 2 ml PBS, given by intravenous inoculation (Williams et al., 2000). Control animals were inoculated

with uninfected Vero cell cultures purified in the same way as the *N. caninum* infected cultures in 2 ml PBS. The inoculum was given within 1 hour of purifying the parasites. Viability was checked by re-infection of the cell monolayer with tachyzoites at the same time as the infection was carried out.

*3.3.1.4 Clinical monitoring and collection of blood samples*

Cattle were observed daily throughout the experimental period. Blood samples were collected from all animals by jugular venipuncture into non-heparinized and heparinized evacuated tubes (Vacutainers, BD Ltd, Oxford, UK) before inoculation and then weekly for the duration of the experiment. Non-heparinized samples were allowed to clot overnight at 4°C before centrifugation at 1000 g for 10 min and removal of serum, which was then divided into aliquots and stored at −20°C until required. Heparinized blood samples were used to collect the peripheral blood mononuclear cells by density gradient centrifugation (see section 3.2.2.2).

*3.3.1.5 Collection of tissue samples*

Immediately after euthanasia, the uterus was removed from each animal and the foetus recovered. Samples from ten randomly selected placentomes with attached inter-placentome area and uterine wall were collected from each uterus. Tissue samples from maternal spleen and uterine (sub-iliac) lymph node (LN) were also collected. Foetal tissue samples collected included the brain and spinal cord, an apical section of the heart, the lung, the main lobe of the liver, the spleen, the left kidney and adrenal gland, the pancreas, a section of jejunum, the quadriceps skeletal muscle, the femoral nerve, the umbilical cord, the bone marrow (BM) from the femur, the thymus, and the mesenteric lymph node (MLN).

From all of these tissues, one sample was placed into 4 % buffered paraformaldehyde (pH 7.4, see Appendix 1) for histological examination and a further sample collected aseptically and frozen at –20 °C for PCR examination. In addition, samples from placentomes and maternal LN were frozen for immunohistology (see 3.2.7.2). Samples of amniotic and allantoic fluid were collected in a sterile manner using a 19 g needle and 2 ml syringe prior to opening the uterus, and frozen for PCR examination. Finally, a small section (<2 mm) of placentome and foetal liver from each animal was fixed in 4 % paraformaldehyde + 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4, see Appendix 1) for transmission electron microscopy.

### 3.3.2 Immune responses of cattle post infection

#### *3.3.2.1 Antibody detection ELISA*

Blood samples were collected by jugular venipuncture prior to challenge and then weekly for the duration of the experiment. Clotted blood was centrifuged at 1000 g for 10 min and serum aliquots were stored at –20 °C. *N. caninum* specific antibody titres were measured using the Mastazyme ELISA (Mast Diagnostics, Liverpool, UK) according to the manufacturer's instructions. This test consists of whole formalin fixed *N. caninum* tachyzoites coated onto a microtitre plate. When diluted bovine serum was added, any specific antibodies to *N. caninum* present bound to the tachyzoites and were detected using a monoclonal antibody specific for bovine IgG conjugated to horseradish peroxidase. The reaction was quantified using the substrate hydrogen peroxidase (0.0075%) and the chromogen tetramethylbenzidine

(TMB) and stopped using hydrochloric acid. The optical densities were read at 450nm using an automated ELISA reader (Dynex Technologies, Revelation, 3.2). To minimise daily and plate variation, and to allow comparison between tests, the optical density readings were expressed as a percentage of a high positive control (percent positivity (PP)). A PP value ≥20 indicated a positive result (Williams et al., 1999).

*3.3.2.2 Peripheral blood mononuclear cell (PBMC) proliferation assay*

Blood samples were collected weekly by jugular venipuncture into heparinized vacutainers prior to challenge and then weekly following infection until the week of euthanasia. PBMC were isolated, as previously described (Williams et al., 2000). A density gradient centrifugation separation technique was carried out using Lymphoprep (Nycomed, Roskilde, Denmark). Briefly, 10 ml blood was mixed with 10 ml 0.9 % NaCl, which was then layered over 20 ml Lymphoprep carefully avoiding any mixing. This was centrifuged at 1000 g for 30 min for the red blood cells to sediment to the bottom of the tube and the PBMC to collect at the interface of the Lymphoprep and plasma. The PBMC were aspirated using a sterile Pasteur pipette and diluted to a 20 ml volume with RPMI 1640 medium supplemented with 10 U/ml heparin sodium (Multiparin®, CP Pharmaceuticals, Wrexham, UK). Following centrifugation at 400 g for 10 min, the cell pellet was suspended in 10 ml RPMI 1640 medium supplemented with 10 U/ml heparin sodium. Cells were counted using an improved Neubauer haemocytometer. Viable PBMC were cultured in RPMI 1640 medium containing 10% foetal calf serum, and penicillin/streptomycin (100 µg/ml; Bio-Whittaker, Walkersville, USA) at a concentration of $2 \times 10^6$ cells/ml, with a final volume of 200 µl. Cells

were stimulated with the non-specific mitogen Concanavalin A (ConA) at 1 μg/ml concentration, *N. caninum* antigen at 2.5 μg/ml concentration, or were unstimulated in medium alone. Each test was performed in triplicate in 96 well flat bottom plates (Nunc, Roskilde, Denmark). Cells were incubated for 5 days at 37 °C and 5% $CO_2$. After this time, the cells were pulsed with 20 μl of $^3H$ Thymidine (Amersham International, Amersham, UK) and incubated at 37 °C and 5 % $CO_2$ for 5 h. Cells were harvested onto glass microfibre filter mats using an automated cell harvester and counted on a micro beta cell counter. The results were expressed as a ratio of the mean counts per minute of test samples to the mean counts per minute for the medium control (stimulation index).

*3.3.2.3 IFNγ ELISA*

PBMC were purified as described in section 3.2.2.2 and cultured as 1 million cells/ml at a final volume of 2 ml in 24-well plates (Nunc, Roskilde, Denmark). Cultured cells were stimulated and incubated as described in section 3.2.2.2. After 3 days the cells were resuspended and supernatants obtained by centrifugation at 300 g for 5 min. Supernatants were stored at –20 °C for IFNγ ELISA.

A commercial ELISA kit (BOVIGAM γ-interferon test kit, Pfizer, Melbourne, Australia) was used to measure levels of IFNγ, according to the manufacturer's instructions. The kit consists of 96 well microtitre plates coated with monoclonal anti-bovine IFNγ, which binds to IFNγ in the sample added. Bound IFNγ was then detected using horseradish peroxidase (HRP)–conjugated monoclonal anti-bovine IFNγ antibody and $H_2O_2$ as the substrate and tetramethylbenzidine (TMB) as the chromogen. The enzyme reaction was stopped using sulphuric acid.

Recombinant bovine IFNγ (1.03 ng/ml, Ciba-Geigy, Basel, Switzerland) was used as a standard for quantitative determinations and was diluted to give standards of 5 ng/ml, 2.5 ng/ml, 1.25 ng/ml, 0.75 ng/ml and 0.375 ng/ml. Each sample was tested in duplicate without dilution and at a 1/20 dilution. In samples with high levels of IFNγ, the test was repeated at a 1/50 and 1/100 dilution. The optical densities were read at 450nm using an automated ELISA reader (Dynex Technologies, Revelation, 3.2). IFNγ concentrations were obtained by linear regression analysis using the recombinant bovine IFNγ standard curve.

### 3.3.3 Histopathological examination

*3.3.3.1 Tissue processing*

After fixation in PFA for 5 days, samples were trimmed into cassettes for processing. For each animal, transverse sections were prepared from all 10 placentomes, to include the adjacent inter-placentome area and uterine wall. Lymph nodes were sectioned in the transverse plane through the hilus. From the foetuses, brain was sectioned to include transverse sections of frontal cerebrum, occipital cerebrum, hippocampus and midbrain, as well as cerebellum and medulla. Transverse sections were prepared from cervical, thoracic, lumbar and sacral segments of the spinal cord. For all other tissues as detailed in section 3.2.1.5, sections from representative portions of each tissue were prepared. After trimming, the samples were processed through graded alcohols and a xylene step, before being routinely embedded in paraffin wax at the Histology Laboratory, Faculty of Veterinary Science. Sections (3-5 μm thick) were cut, dried overnight

at 37 °C, stained with haematoxylin and eosin (HE) and examined for any pathological changes.

*3.3.3.2 Haematoxylin and eosin (HE) staining*

The staining was performed by the technical staff in the Histology Laboratory, Faculty of Veterinary Science. Following deparaffination in xylene and rehydration through graded alcohols, sections were placed in Mayers haemalaun (see Appendix 1) for 5 min, then blued in running tap water for 6 min. Sections were then stained in a working solution of eosin (see Appendix 1) for 2 min. Slides were dehydrated in 95 % alcohol for 7 dips and then submerged for 1 min, which was repeated 3 times. The process of 7 dips and then submerged for 1 min was repeated 3 times with absolute alcohol. Sections were cleared in xylene and mounted in DPX (BDH brand, VWR International, Lutterworth, UK).

### 3.3.4 Immunohistological demonstration of *N. caninum*

*3.3.4.1 Preparation of a positive control*

*N. caninum* infected Vero cell cultures were produced as described in section 3.2.1.3, for use as a positive control. Eight 80 $cm^2$ cell culture flasks were harvested using a cell scraper and centrifuged at 1000 g for 10 min. Following suspension in 2 ml RPMI medium and centrifugation at 1000 g for 10 min the cell pellet was resuspended in 4 % buffered paraformaldehyde (PFA). Following a further centrifugation step at 1000 g for 10 min, the pellet was fixed for 24 h in PFA and was then processed as described in section 3.2.3.1. 3-5 µm sections mounted on poly-L-lysine coated slides were used for immunohistology.

*3.3.4.2 Immunohistological protocol*

For each section that was examined histologically, a subsequent 3-5 μm section was cut, mounted onto a poly-L-lysine coated slide, and examined immunohistologically for the presence of *N. caninum* antigen. The peroxidase-anti-peroxidase (PAP) method was applied (Kipar et al., 1998). Sections were incubated in methanol with 0.5 % $H_2O_2$ (Perhydrol 30 %, Fisher Scientific, Loughborough, UK) for 30 min to inactivate endogenous peroxidase, following deparaffination in xylene and rehydration through graded alcohols. Slides were placed in coverplates in Sequenza racks (Thermo Shandon, Pittsburgh, USA). Following a 5 min Tris buffered saline (TBS, pH 7.4, see Appendix 1) wash, non-specific binding of antiserum was blocked by incubation in 50 % swine serum in TBS for 10 min. Slides were incubated for 15-18 h at 4 °C with a polyclonal rabbit anti-*N. caninum* antibody (Barber et al., 1995). This dilution was optimised to 1:1500 in 20 % swine serum in TBS, by trying a range of dilutions and opting for the dilution that gave a positive reaction with minimal background staining in the positive control sections. For each section, a subsequent section was stained using normal rabbit serum at the same dilution as a negative control. The slides were washed for 5 min in TBS then incubated for 30 min with swine anti-rabbit IgG at 1:100 dilution in 20 % swine serum in TBS. A further 5 min TBS wash was followed by 30 min incubation with rabbit PAP complex at 1:100 dilution in 20 % swine serum in TBS. Slides were then incubated with stirring for 10 min with 0.5 mg/ml 3,3' diaminobenzidine tetrahydrochloride (DAB, Fluka, Buchs, Switzerland) with 0.01 % $H_2O_2$ (Perhydrol 30 %, Fisher Scientific) in 0.1 M imidazole buffer (pH 7.1, see Appendix 1). Slides were counterstained for 30 sec in Papanicolaou's

haematoxylin (see Appendix 1), placed in running tap water for 5 min, dehydrated in ascending alcohols, cleared in xylene and mounted. Unless stated otherwise, all sera and antibodies used in this protocol were from Dakocytomation, Glostrup, Denmark.

### 3.3.5 Detection of *N. caninum* by polymerase chain reaction (PCR)

#### *3.3.5.1 Culture of tachyzoites for positive control*

Extracted DNA from purified *N. caninum* tachyzoites was used as a positive control for all PCR reactions. Vero cell cultures were established using methods described previously (Williams *et al.*, 2000). *N. caninum* tachyzoites were cultured and harvested as described in section 3.2.1.3. The number of tachyzoites collected was calculated using a Fuchs Rosenthal counting chamber. A final number of $10^6$ tachyzoites in 100 µl PBS was used to extract *N. caninum* DNA using the method detailed in chapter 2.

#### *3.3.5.2 Extraction of DNA*

DNA was extracted from 3 placentomes, 3 interplacentome areas, foetal tissues including brain, spinal cord, heart, liver, lung, skeletal muscle and kidney, as well as allantoic fluid and amniotic fluid from each animal using the DNeasy® tissue extraction kit (Qiagen Ltd, Crawley, UK), following the manufacturer's instructions for animal tissues. This kit uses advanced silica-gel-membrane technology for rapid purification of total cellular DNA. Samples were lysed with proteinase K and buffering conditions were adjusted to provide optimal binding conditions before the lysate was loaded onto the DNeasy® mini spin column. During centrifugation, DNA was selectively bound to the DNeasy® membrane.

Contaminants and enzyme inhibitors were removed by 2 wash steps, and DNA was eluted into 100 µl of buffer, ready for use. For each solid tissue 25 mg was taken and used to extract DNA. In the case of amniotic and allantoic fluid samples, 200 µl was used. DNA was extracted from $10^6$ tachyzoites in 100 µl PBS as a positive control (see 3.2.5.1) and DNA from 25 mg of brain from a newborn calf serologically negative for *N. caninum* was used as a negative control.

*3.3.5.3 Reagent concentrations for PCR*

In the first PCR, a sample volume of each 3 µl was used, and 2 µl in the second PCR. The samples for the second reaction were handled in a room separate from the first reaction in order to minimise the risk of contamination. The 50 µl reaction mixtures used for each step contained the sample, 200 µM of each deoxynucleotide, Hot star Taq (Qiagen Ltd, Paisley, UK, 2.5 units per reaction), and buffer with a final concentration of 1.5 mM $MgCl_2$. Primer concentrations were 0.1 µM and 0.4 µM in reactions 1 and 2, respectively. Cultured *N. caninum* tachyzoite DNA and ultrapure water were used as positive and negative controls respectively.

*3.3.5.4 Oligonucleotide primer sequences for PCR*

The primers used were from the method of Uggla et al. (1998), which was found in chapter 2 to be the more sensitive method in our laboratory. The external primers, F6 and 5.8B, 5'- CAG GTC TGT GAT GCC C-3' and 5'-TCG CGT TTT GCT GCG TTC TTC-3', respectively, were complementary to regions of the rDNA unit (18S and 5.8S), and used in the first PCR. The internal primers

PN3 and PN4, 5′-TAC TAC TCC CTG TGA GTT G-3′ and 5′-TCT CTT CCC TCA AAC GCT A-3′, respectively, specific for *N. caninum*, were complementary to the ITS1 region, and were used in the second PCR. (Primers from Sigma Genosys, Dorset, UK).

*3.3.5.5 Reaction conditions for PCR*

Initially the samples were heated for 2.5 min at 94 °C; thereafter each cycle consisted of a 30 sec denaturation at 94 °C, 40 sec at the annealing temperature of 50 °C, followed by a 1 min extension at 72 °C. Amplification was performed with F6 and 5.8B over 25 cycles, followed by a final extension at 72 °C for 3 min. In the second PCR the annealing temperature was increased to 54 °C, extension decreased to 30 sec and the amplification was performed with PN3 and PN4 over 30 cycles, as above.

*3.3.5.6 Agarose gel electrophoresis*

10 µl aliquots of reaction mixtures were analysed by 2 % agarose gel electrophoresis and the products stained with SYBR safe® DNA gel stain (Invitrogen, Paisley, UK) and visualised under U.V. light.

**3.3.6 Detection of *N. caninum* by transmission electron microscopy (TEM)**

Tissue samples, cut into approximately 2 $mm^3$ blocks, were stored in 5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4 °C until processing. Samples were then washed in 0.1 M cacodylate buffer and postfixed in 1 % (w/v) osmium tetroxide for 75 min. Following a further wash in 0.1 M cacodylate buffer, samples were incubated in uranyl acetate for 90 min, changing the

medium every 30 min. Samples were dehydrated in graded ethanol, transferred into acetone and incubated in a 50 % (w/v) resin mix (see Appendix 1) and 67 % (w/v) resin mix in acetone for 1 h each. They were then transferred into moulds of undiluted resin mix for embedding at 60 °C for 15-18 h. Semi-thin sections were cut, stained with methylene blue and examined to select areas of interest (focal necrosis). 90 nm ultrathin sections were cut from polymerised blocks with a diamond knife (Diatome Ltd., Biel, Switzerland) on an ultracut ultramicrotome (Reichert Jung, Vienna, Austria). Sections were transferred onto 150-mesh copper grids, stained with Reynold's lead citrate for 5 min, and viewed in a H-600 transmission electron microscope (Hitachi, Pleasanton, California, USA).

### 3.3.7 Detection of infiltrating leukocytes in the placenta by immunohistology

#### *3.3.7.1 Immunohistology on fixed tissues*

For each animal, two randomly selected placentomes were taken and immunohistology carried out to identify any inflammatory or immune cells present. Details of the antibodies used and the leukocyte populations that they are directed against are shown in Table 3.3.2.

**Table 3.3.2 - Antibodies used to detect leukocyte subsets in the placenta**

| Ligand | Antibody | Cells marked |
|---|---|---|
| CD3 | Polyclonal rabbit anti-human CD3 | T cells |
| CD79α | Monoclonal mouse anti-human CD79α (clone JCB117) | B cells |
| Myeloid/ histiocyte antigen | Monoclonal mouse anti-human myeloid/histiocyte antigen (clone MAC387) | Macrophages and neutrophils |

The PAP and avidin biotin complex methods were applied (Kipar et al., 1998; Hsu et al., 1981). Sections were placed in methanol with 0.5 % $H_2O_2$ for 30 min to inactivate endogenous peroxidase, following deparaffination in xylene and rehydration through graded alcohols. Sections were pre-treated with the antigen retrieval method optimised for the primary antibody as detailed in Table 3.3.3.

**Table 3.3.3 – Antigen retrieval methods**

| Antibody | Antigen retrieval method | Details of method |
|---|---|---|
| CD3 | Enzymatic digestion | Incubation in pre-warmed phosphate buffered saline (PBS, pH 7.2, see Appendix 1) at 37 °C for 5 min, followed by incubation in PBS with 0.05 % protease (type XXIV, bacterial, VWR International, Lutterworth, UK) at 37 °C for 5 min. 5 min wash with ice cold TBS. |
| CD79α | Microwave irradiation | Irradiation in scientific microwave oven (Samsung TDS) at maximal power of 800 W for 10 min in 10mM EDTA (pH 9, see Appendix 1). 5 min wash in TBS. |
| Myeloid/ histiocyte antigen | Enzymatic digestion | As CD3 |

Slides were washed for 5 min in TBS, then placed in coverplates in Sequenza racks. Following a 5 min TBS wash, non-specific binding of antiserum was blocked for 10 min in the blocking agent (see Table 3.3.4). Slides were then incubated for 15-18 h at 4 °C with the primary antibody. For each section, a subsequent section was stained using normal or isotype specific serum from the species in which the primary antibody was produced (negative control). The slides were washed for 5 min in TBS then incubated for 30 min with the secondary antibody. A further 5 min TBS wash was followed by 30 min incubation with the tertiary antibody, with the exception of the Vectastain ABC kit.. Details and dilutions of the blocking agents and antibodies are shown in Table 3.3.4.

**Table 3.3.4 – Detection systems of antibodies used to stain leukocyte subsets in the placenta**

| Antibody | Blocking agent | Primary antibody | Secondary antibody | Tertiary antibody |
|---|---|---|---|---|
| CD3 | 1:2 swine serum in TBS | 1:5 in 20 % swine serum in TBS | Swine anti-rabbit IgG 1:100 dilution in 20% swine serum in TBS | Rabbit PAP complex 1:100 dilution in 20 % swine serum in TBS |
| CD79a | undiluted horse serum | 1:50 in TBS | Vectastain ABC kit (Vector Laboratories Ltd, Peterborough, UK) | |
| | | | Biotinylated horse anti-mouse IgG 1:100 in TBS | Avidin and Biotin 1: 100 each in TBS |
| Myeloid/ histiocyte antigen | 1:10 rat serum in TBS | 1:1000 in TBS | Rat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA, USA) 1:100 in TBS | Mouse PAP complex (Jackson Immunoresearch, Laboratories, West Grove, PA, USA) 1:500 in TBS |

Slides were then incubated with stirring for 10 min with 0.5 mg/ml 3,3' DAB with 0.01 % $H_2O_2$ in 0.1 M imidazole buffer (pH 7.1). Sections were counterstained for 30 sec in Papanicolaou's haematoxylin, placed in running tap water for 5 min, dehydrated in ascending alcohols, cleared in xylene and mounted with DPX. A section of bovine lymph node was used as a positive control for each antibody, again also with corresponding negative control. Unless

stated otherwise, all sera and antibodies used in this protocol were from Dakocytomation, Glostrup, Denmark.

*3.3.7.2 Immunohistology using frozen sections to characterise T cells in the placenta*

For each animal, two placentomes were randomly selected and immunohistology carried out on frozen sections to further characterise the T cells present. The placentomes used were collected and placed on ice. Within 5 h, 5 $mm^3$ blocks of the placentomes were prepared by transverse section to include the attached inter-placentome area and uterine wall. Tissues were embedded in Tissue-Tek OCT compound (Pelco International, Redding, CA, USA) wrapped in an aluminium foil tube and frozen by immersion in isopentane (VWR International, Lutterworth, UK) chilled in liquid nitrogen. The frozen tissue blocks were stored at –70 °C until sectioning.

Sections, 10 µm in thickness, were cut on a cryostat and placed onto poly-L-lysine coated slides. After being air dried for 10 min, sections were fixed in 100% ice cold acetone for 10 min and air dried again for 10 min before storage at –70°C. Before staining, sections were allowed to thaw to room temperature for 2 h. Sections were then placed in methanol with 0.5 % $H_2O_2$ for 15 min to inactivate endogenous peroxidase. Slides were placed in TBS for 5 min, then were placed in coverplates in Sequenza racks. Following a 5 min TBS wash, non-specific binding of antiserum was blocked by incubation with undiluted horse serum for 10 min. Slides were incubated overnight at 4 °C with one of the antibodies shown in Table 3.3.5.

**Table 3.3.5 - Monoclonal antibodies for detection of T - lymphocyte subsets in the placenta**

| Ligand | Antibody |
|---|---|
| CD4 | Monoclonal mouse anti-bovine CD4 (clone IL-A11, EAECC) |
| CD8 | Monoclonal mouse anti-bovine CD8 (clone IL-A105, EAECC) |

The ABC method was applied (Hsu et al., 1981). The dilution of both antibodies was optimised to 1:10 in TBS by trying various dilutions and opting for the dilution with a positive reaction and the least background staining. For each section, a subsequent section was stained using isotype specific mouse serum at the same dilution (negative control). The slides were washed for 5 min in TBS then incubated for 30 min with biotinylated horse anti-mouse IgG at 0.9:100 dilution in TBS. A further 5 min TBS wash was followed by 30 min incubation with avidin and biotin (Vectastain ABC kit), each at 1:100 dilution in TBS. Slides were then incubated with stirring for 10min with 3,3' DAB with 0.01 % $H_2O_2$ in 0.1 M imidazole buffer (pH 7.1), counterstained for 30 sec in Papanicolaou's haematoxylin, placed in running tap water for 5 min, dehydrated in ascending alcohols, cleared in xylene and mounted with DPX. A section of bovine lymph node was used as a positive control for each antibody, again also with corresponding negative control. Unless stated otherwise, all sera and antibodies used in this protocol were from Dakocytomation, Glostrup, Denmark.

### 3.3.8 Detection of interferon gamma (IFN-γ) in the placenta by immunohistology

The PAP method was applied (Kipar et al., 1998). Sections were prepared from PFA-fixed and paraffin-embedded tissue. Interferon gamma was identified in two randomly selected placentomes from a group 1 animal by immunohistology. The primary antibody used was a monoclonal mouse anti-bovine interferon gamma (clone 7B6; Serotec, Oxford, UK). This antibody was used at a dilution of 1:50 in TBS following antigen retrieval in citrate buffer pH6 (see Appendix 1) for 30 min in a water bath at 97 °C. Otherwise the protocol was as described for myeloid/histiocyte antigen in section 3.3.7.1.

## 3.4 Results

### 3.4.1 Foetal death occurred in cattle infected with *N. caninum* at 70 dg but not at 210 dg.

Re-infection of cell monolayers with tachyzoites on the day of infection confirmed that the parasite inocula were viable. Cattle inoculated with parasites at 70 dg were examined three times a week to monitor foetal viability by trans-rectal ultrasonography until day 14 post challenge and daily thereafter. Foetal death occurred in all animals infected at 70 dg (group 1), 22.7±1.2 days after infection and animals were euthanased within 24 h of detection of foetal death by the absence of a foetal heartbeat on ultrasound examination. One animal (I2) had twin foetuses. Cattle inoculated with parasites at 210 dg were examined three times a week to monitor foetal viability by trans-abdominal ultrasonography. Foetal death did not occur in the cattle inoculated at 210 dg (group 3), and these cattle were killed between 20 and 22 days post challenge. All foetuses from group 3 cattle were alive on the morning of euthanasia. All control animals (groups 2 and 4) were monitored three times a week for foetal viability. No problems were detected with any of the foetuses in the control groups and all control foetuses were alive on the morning of euthanasia. Animals were killed by captive bolt pistol and dams and foetuses were subjected to a post-mortem examination. The timing of foetal death and sample collection for all animals is summarised in Table 3.4.1.

**Table 3.4.1 – Outcome of infection.**

| *Cattle Group* (type and timing of inoculums) | Individual animal numbers | Day of foetal death (dpi) | Day of sample collection (dpi) |
|---|---|---|---|
| **1** ($10^7$ *N. caninum* tachyzoites at 70 dg) | I1 | 19 | 21 |
| | I2 | 19 | 21 |
| | I3 | 25 | 25 |
| | I4 | 25 | 25 |
| | I5 | 20 | 20 |
| | I6 | 26 | 26 |
| **2** (uninfected Vero cells at 70 dg) | C1 and C2 | Not applicable (N/A) | 20 |
| | C3 and C4 | N/A | 21 |
| | C5 and C6 | N/A | 22 |
| **3** ($10^7$ *N. caninum* tachyzoites at 210 dg) | I7 and I8 | N/A | 20 |
| | I9 and I10 | N/A | 21 |
| | I11 and I12 | N/A | 22 |
| **4** (uninfected Vero cells at 210 dg) | C7 and C8 | N/A | 20 |
| | C9 and C10 | N/A | 21 |
| | C11 and C12 | N/A | 22 |

### 3.4.2 Animals seroconverted by two weeks post-infection and PBMC proliferated and secreted IFN-γ in response to *N. caninum* antigen from one week post-infection.

*3.4.2.1 Antibody detection ELISA*

Prior to infection, all animals were sero-negative for *N. caninum* (PP<20). Animals in the control groups (groups 2 and 4) remained sero-negative for the duration of the experiment (Figs. 3.4.2 and 3.4.4). In group 1, 1/6 animal (I5) had sero-converted (PP>20) by 7dpi and all animals had sero-converted by 14dpi (Fig. 3.4.1). In group 3, 5/6 animals sero-converted by 14dpi (Fig. 3.4.3). The remaining animal (I11) had not sero-converted at the time of euthanasia. However, this animal had increasing antibody levels post infection (mean PP1.7 prior to infection, rising to 8 at 21dpi). The mean PP values for the animals infected at 210 dpi was significantly lower than the animals infected at 70 dpi at one time-point of 3 weeks post infection. (P= 0.0021 using an unpaired t test, see Fig. 3.4.12A).

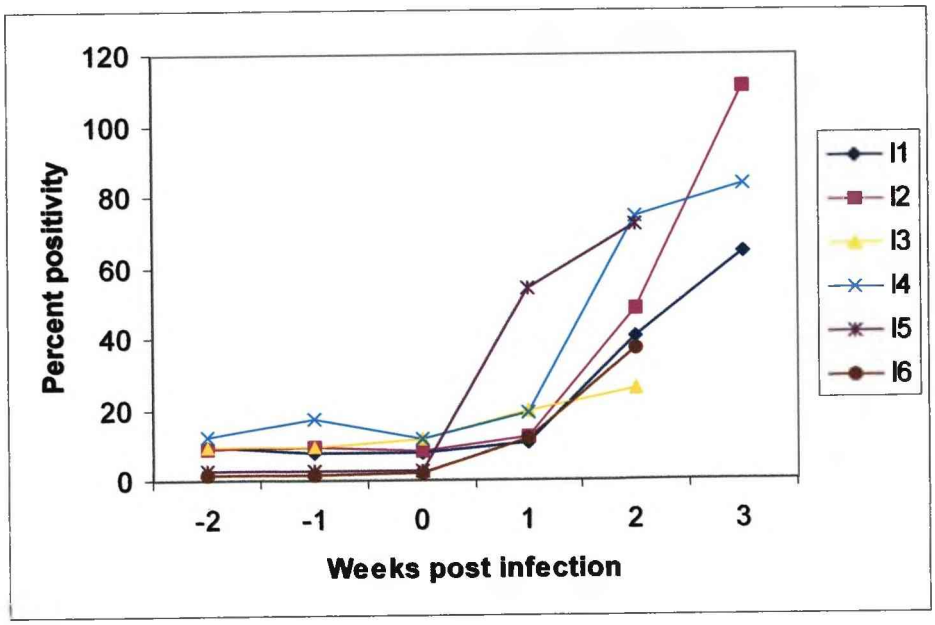



**Figure 3.4.1:** Immune responses in six pregnant cattle challenged with $10^7$ *N. caninum* tachyzoites at 70 days gestation; *N. caninum* specific antibody responses in an ELISA shown as percent positivity (PP) of a positive control serum.

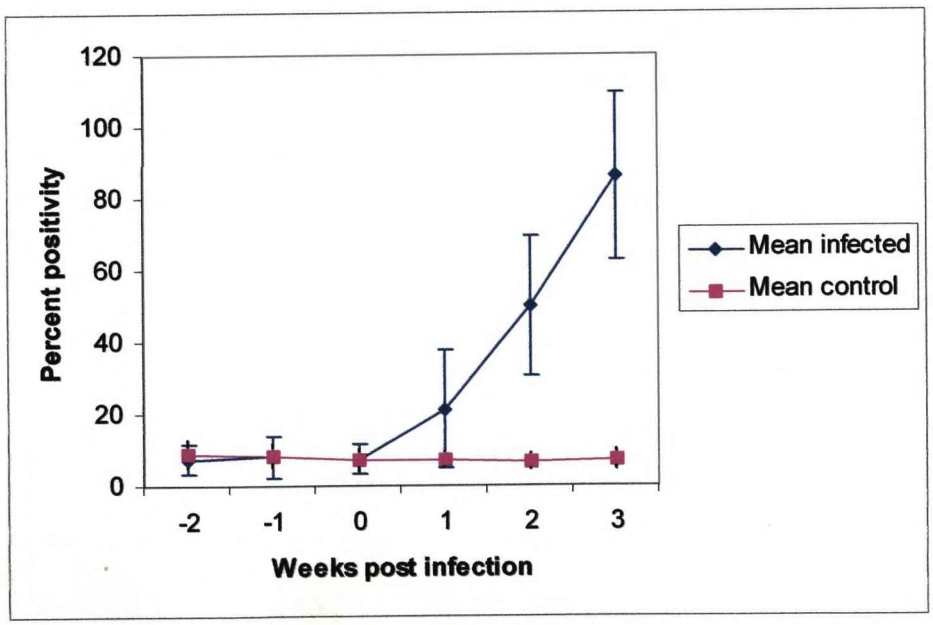



**Figure 3.4.2:** Mean immune responses in six pregnant cattle challenged with $10^7$ *N. caninum* tachyzoites at 70 days gestation and six control animals; *N. caninum* specific antibody responses in an ELISA shown as percent positivity (PP) of a positive control serum.

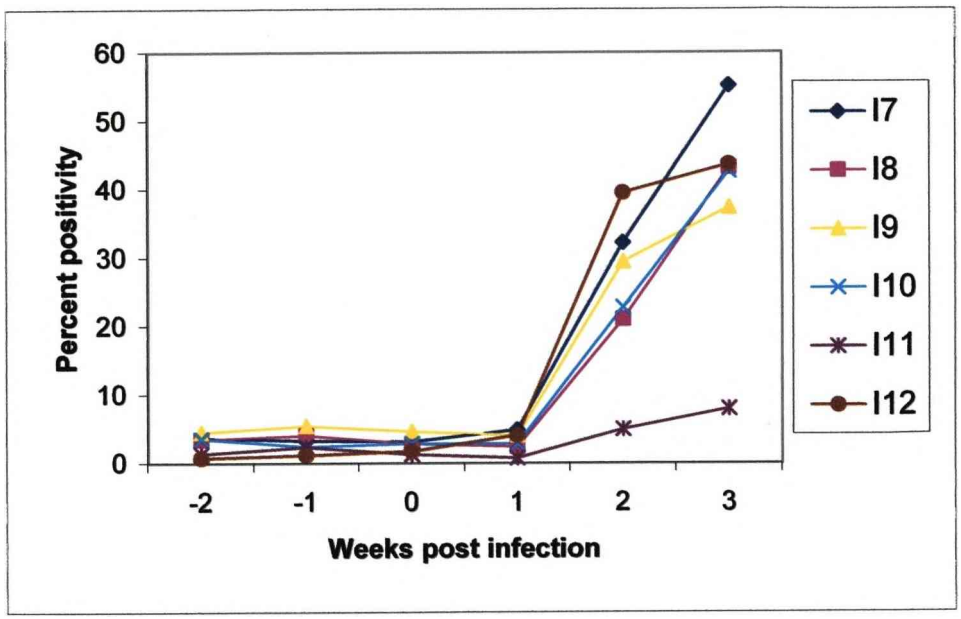



**Figure 3.4.3:** Immune responses in six pregnant cattle challenged with $10^7$ *N. caninum* tachyzoites at 210 days gestation; *N. caninum* specific antibody responses in an ELISA shown as percent positivity (PP) of a positive control serum.

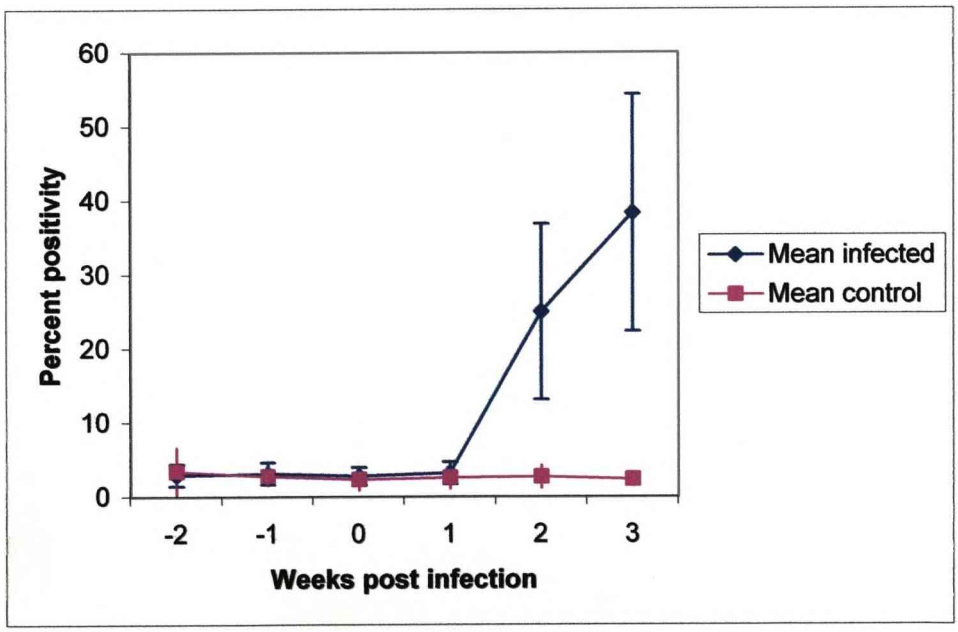



**Figure 3.4.4:** Mean immune responses in six pregnant cattle challenged with $10^7$ *N. caninum* tachyzoites at 210 days gestation and six control animals; *N. caninum* specific antibody responses in an ELISA shown as percent positivity (PP) of a positive control serum.

*3.4.2.2 PBMC proliferation assay*

Blood samples were collected weekly prior to challenge and then weekly for the duration of the experiment and the proliferation of mononuclear cells in response to *N. caninum* measured. Prior to infection, PBMC of all animals did not respond to *Neospora* antigen. Animals in the control groups (groups 2 and 4) did not respond to *Neospora* antigen for the duration of the experiment (Fig. 3.4.5 and 3.4.7). In group 3, all animals where responding to *Neospora* antigen by one week post infection (Fig. 3.4.6). In group 1, a machinery problem led to data only being available for 2 animals, but both were responding to *Neospora* antigen by one week post infection (Fig. 3.4.5).

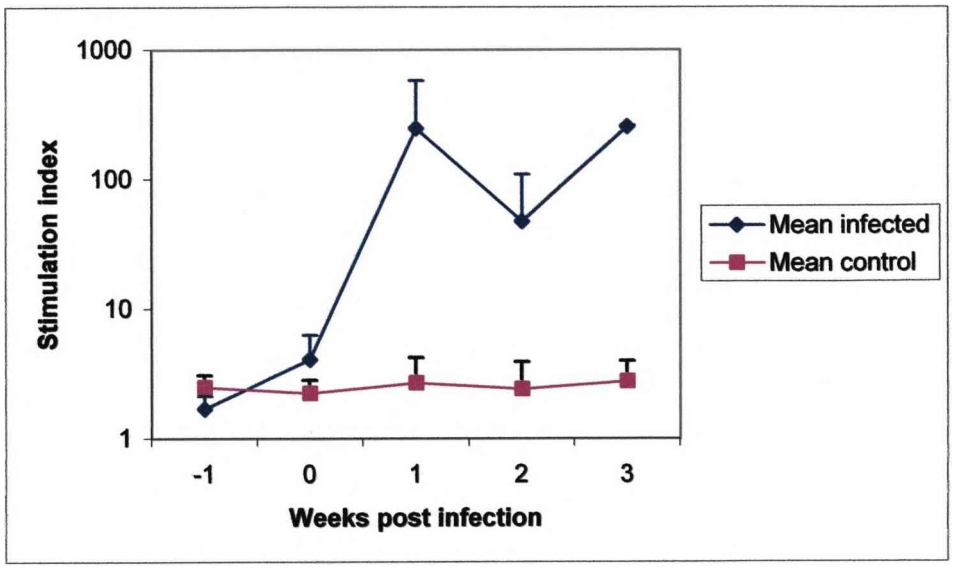



**Figure 3.4.5:** Mean immune responses in two pregnant cattle challenged with $10^7$ *N. caninum* tachyzoites at 70 days gestation and six control animals. Antigen specific proliferation of PBMCs shown as a ratio of the mean counts per minute of test samples to the mean counts per minute of the medium control (stimulation index).

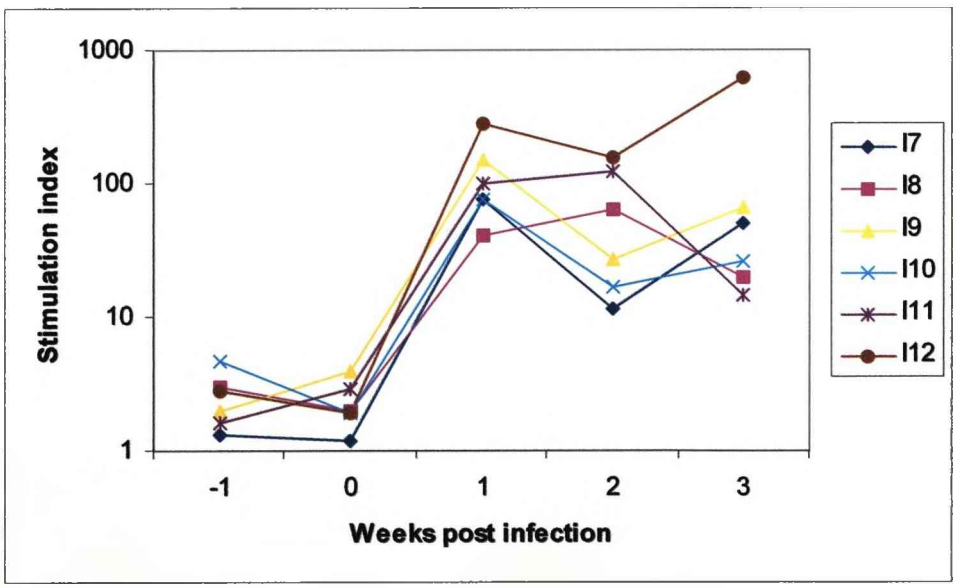



**Figure 3.4.6:** Immune responses in six pregnant cattle challenged with $10^7$ *N. caninum* tachyzoites at 210 days gestation. Antigen specific proliferation PBMCs shown as a ratio of the mean counts per minute of test samples to the mean counts per minute of the medium control (stimulation index).

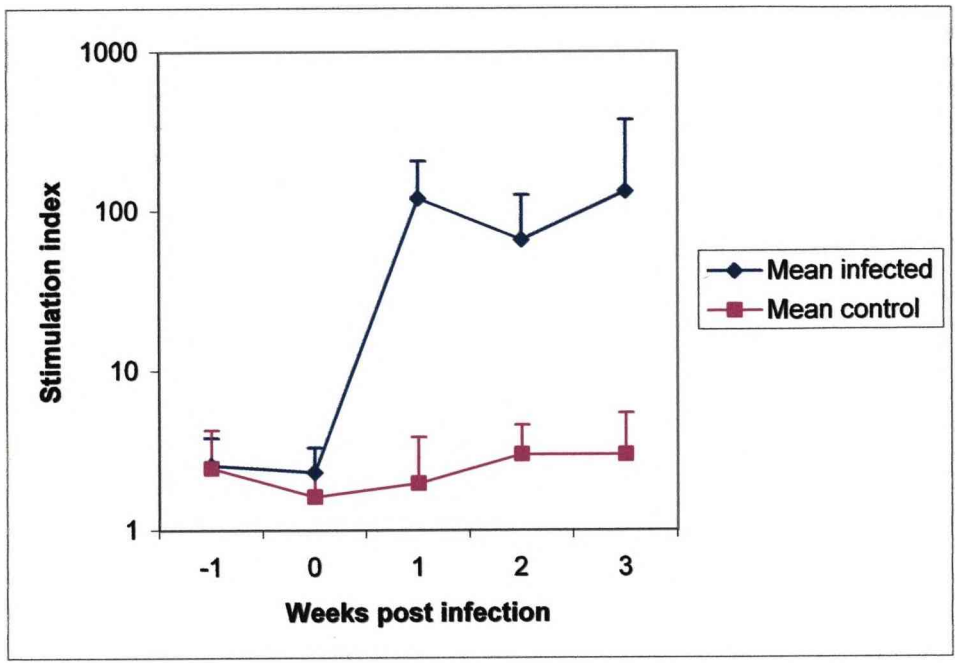



**Figure 3.4.7:** Mean immune responses in six pregnant cattle challenged with $10^7$ *N. caninum* tachyzoites at 210 days gestation and six control animals. Antigen specific proliferation of PBMCs shown as a ratio of the mean counts per minute of test samples to the mean counts per minute of the medium control (stimulation index).

*3.4.2.3 IFNγ ELISA*

Prior to infection, PBMC from all animals did not produce IFNγ in response to *Neospora* antigen. Animals in the control groups (groups 2 and 4) did not respond to *Neospora* antigen for the duration of the experiment (Fig. 3.4.9 and 3.4.11). In group 1, all animals except I2 were responding to *Neospora* antigen by one week post infection (Fig. 3.4.8) and I2 responded by week 4. In group 3, all animals were responding to *Neospora* antigen by one week post infection (Fig. 3.4. 10). The mean IFNγ levels for the two infected groups were not statistically different at any of the time-points (Fig. 3.4.12B).

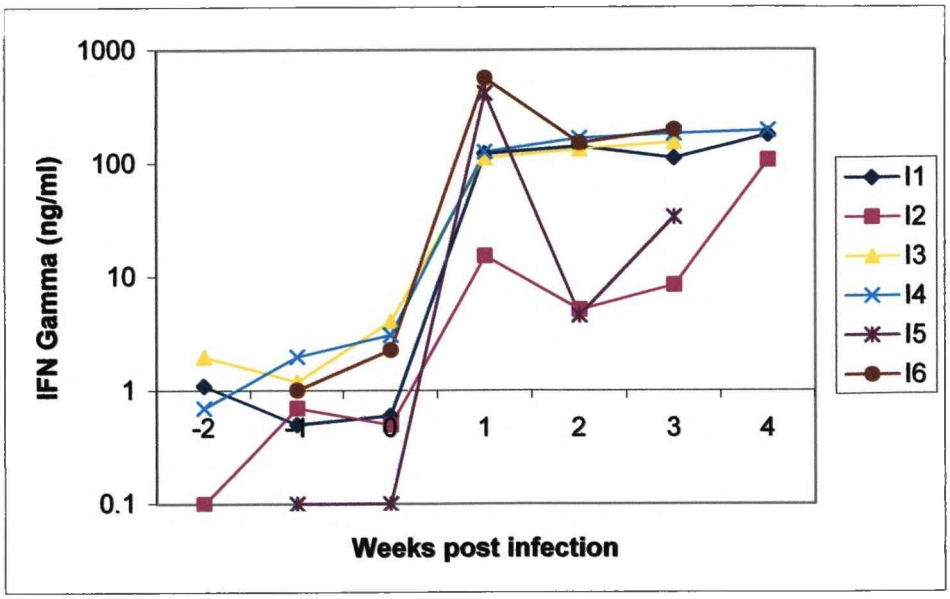



**Figure 3.4.8:** Immune responses in six pregnant cattle challenged with $10^7$ *N. caninum* tachyzoites at 70 days gestation; *N. caninum* antigen specific IFNγ production by PBMCs.

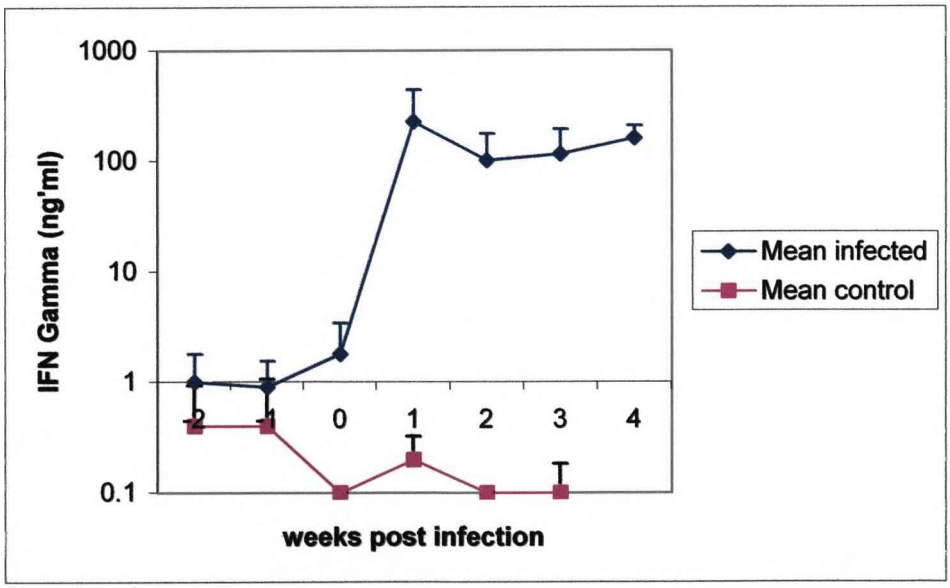



**Figure 3.4.9:** Mean immune responses in six pregnant cattle challenged with $10^7$ *N. caninum* tachyzoites at 70 days gestation and six control animals; *N. caninum* antigen specific IFNγ production by PBMCs.

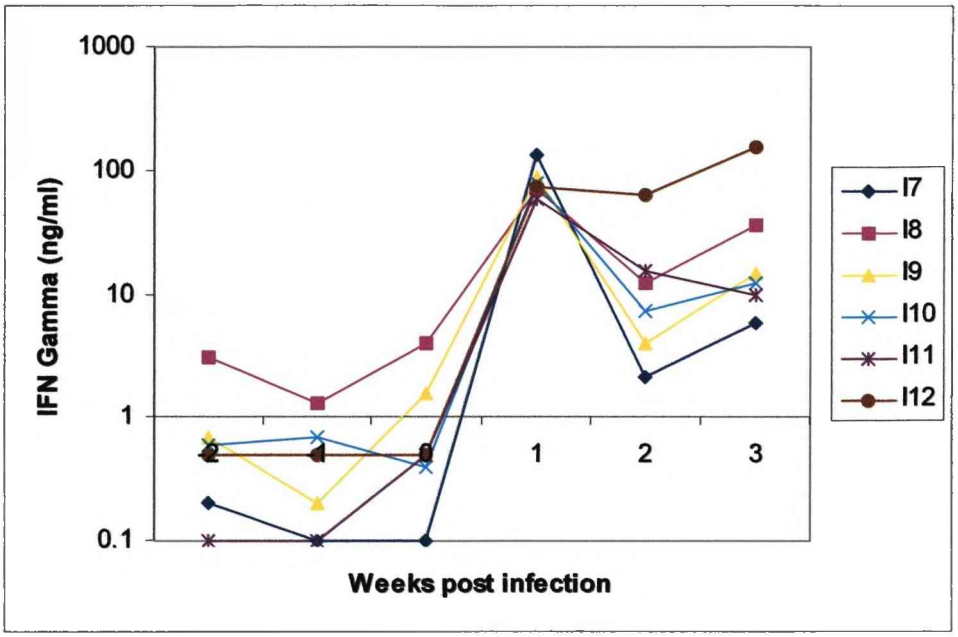



**Figure 3.4.10:** Immune responses in six pregnant cattle challenged with $10^7$ *N. caninum* tachyzoites at 210 days gestation; *N. caninum* antigen specific IFNγ production by PBMCs.

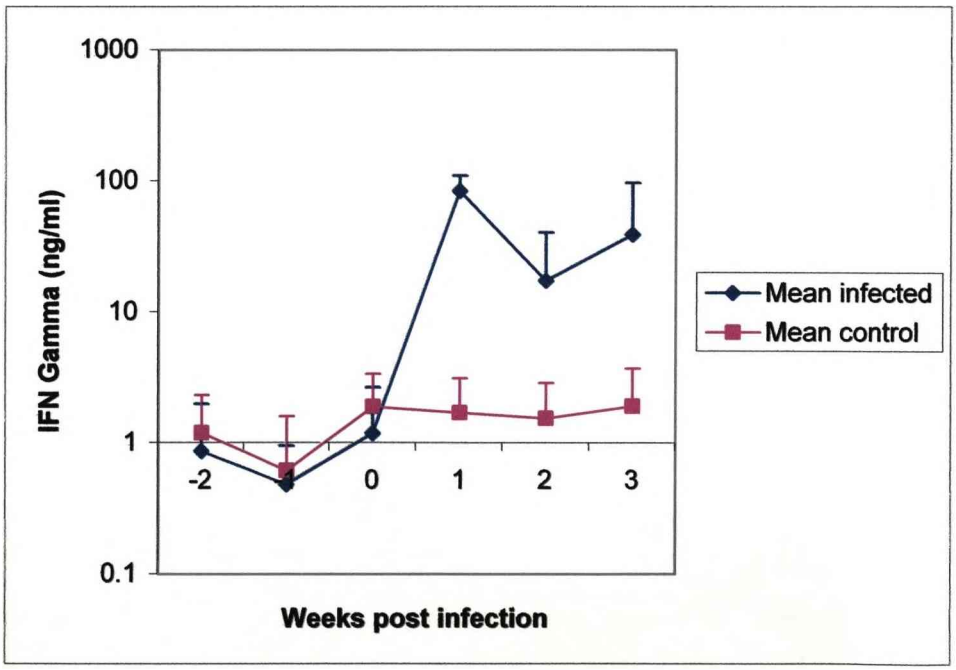
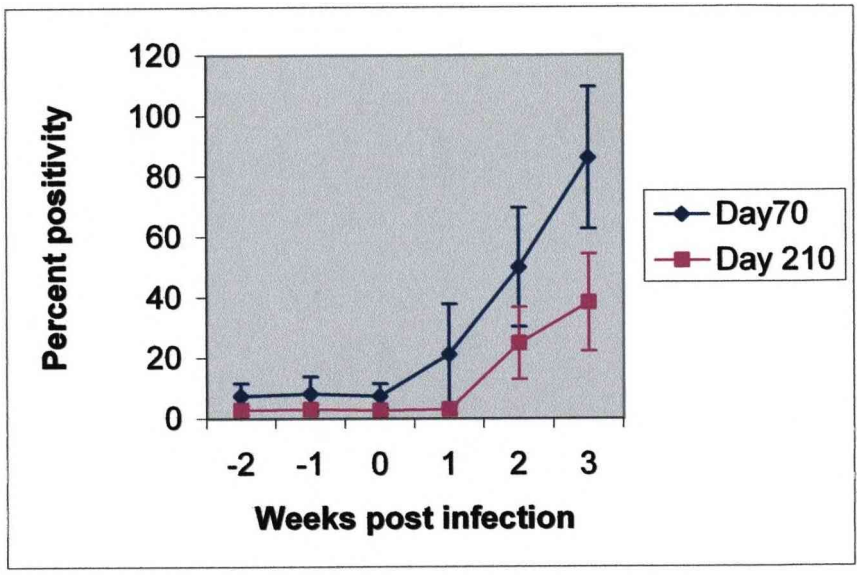
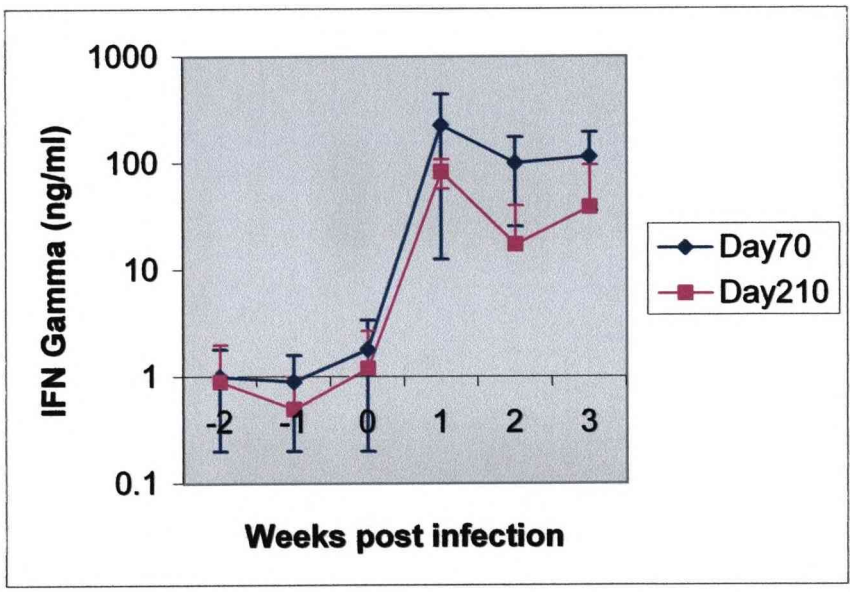



**Figure 3.4.11:** Mean immune responses in six pregnant cattle challenged with $10^7$ *N. caninum* tachyzoites at 210 days gestation and six control animals; *N. caninum* antigen specific IFNγ production by PBMCs.




(A)




(B)

**Figure 3.4.12:** Mean immune responses in six pregnant cattle challenged with $10^7$ *N. caninum* tachyzoites at 70 days gestation and 210 days gestation: (A) *N. caninum* specific antibody responses in an ELISA shown as percent positivity (PP) of a positive control serum (B) *N. caninum* antigen specific IFNγ production by PBMCs.

### 3.4.3 Foetal death is associated with widespread placental necrosis and the presence of parasites within foetal and maternal epithelial cells.

Histopathological examination of the placenta in animals where foetal death had occurred after infection at 70 dg revealed multifocal epithelial cell necrosis in all placentomes examined (n=10 per animal). Both foetal and maternal epithelial cells were affected, ranging from single necrotic epithelial cells to extensive focal necrosis affecting large numbers of foetal villi and surrounding maternal epithelial cells (Fig 3.4.13). Ultrastructural examination identified parasites within degenerating epithelial cells (Figs 3.4.14A, B), cell-free adjacent to areas of cellular debris (Fig 3.4.14B) and cell-free in the maternal interstitial layer. Immunohistology for *N. caninum* antigen stained structures resembling *N. caninum* tachyzoites and granular material, consistent with parasite debris or secreted antigen. Labelling was most intense within and adjacent to areas of necrosis, where antigen was observed both extracellularly and within degenerating cells. Tachyzoites were identified in the cytoplasm of intact and necrotic maternal endometrial and foetal trophoblast epithelial cells (Fig 3.4.15A). Within necrotic areas and in the adjacent tissue, infiltration by macrophages and occasional neutrophils was observed (Fig 3.4.15B). In addition, a moderate, diffuse, lymphocyte-dominated, mononuclear interstitial infiltration was present (Fig 3.4.15C). At the base of the caruncles, mononuclear, lymphocyte-dominated perivascular infiltrates were observed. In most placentomes, focal haemorrhage and/or focal serum leakage was present at the villous tips or in the spaces between the maternal and foetal cell layers. Occasional tachyzoites were identified by immunohistology within circulating leucocytes in maternal blood vessels (Fig.3.4.15D).

After infection at 210 dg, histological alterations were restricted to occasional, small areas of epithelial necrosis, usually affecting one foetal villus and the surrounding maternal epithelium. Where these lesions were present, a mild lymphocyte-dominated mononuclear interstitial infiltration was observed in the maternal tissue. *N. caninum* antigen was not detected by immunohistology.
In the two control groups, neither histological changes nor *N. caninum* antigen were detected in any of the placentomes examined.

**3.4.4 Foetal death is associated with widespread necrosis in foetal tissues.**

Histopathological examination of foetuses that had died after infection at 70 dg revealed extensive necrosis in numerous foetal tissues. Necrosis was most severe in the brain, spinal cord (Fig 3.4.16A) and liver (Fig 3.4.16B) and was multifocal to coalescing. Ultrastructural examination of the liver confirmed that death of hepatocytes occurred via necrosis (Fig 3.4.16C). In other tissues, scattered individual necrotic parenchymal cells were observed. These comprised myocytes in skeletal muscle, renal tubular epithelial cells as well as pulmonary and pancreatic parenchymal cells and cells in thymus, spleen and bone marrow. The remaining tissues (heart, adrenal gland, pancreas, jejunum, femoral nerve and mesenteric lymph node) did not exhibit any histological changes. There was no evidence of an inflammatory cell infiltration in any of the foetal tissues. Immunohistology identified *N. caninum* antigen in all foetuses and tachyzoites were detected in a number of intact and degenerating cells, in particular in glial cells in the brain and spinal cord (Fig 3.4.16D), hepatocytes, skeletal myocytes, renal tubular epithelial cells and leucocytes in spleen and bone marrow. Tachyzoites were found within the cytoplasm of a megakaryocyte in the liver

(Fig. 3.4.16E) and within the cytoplasm of haematopoietic cells in the bone marrow. Clusters of tachyzoites were also observed within single intact cardiac myocytes in 6/7 foetuses (Fig 3.5.16F).

After infection at 210 dg, histopathological changes were restricted to the foetal brain and spinal cord. In one foetus, focal microgliosis and perivascular mononuclear (lymphocytes and macrophages) cell infiltration in the spinal cord was observed (Fig 3.4.17A) and in a second foetus focal encephalomyelitis with a perivascular mononuclear cell infiltration was seen. In both cases, *N. caninum* antigen was detected associated with these lesions (Fig 3.4.17B). All other tissues examined from these two foetuses as well as all tissues from the other four foetuses were negative for parasite antigen.

In the two control groups, neither histological changes nor *N. caninum* antigen were observed in any foetal tissue.

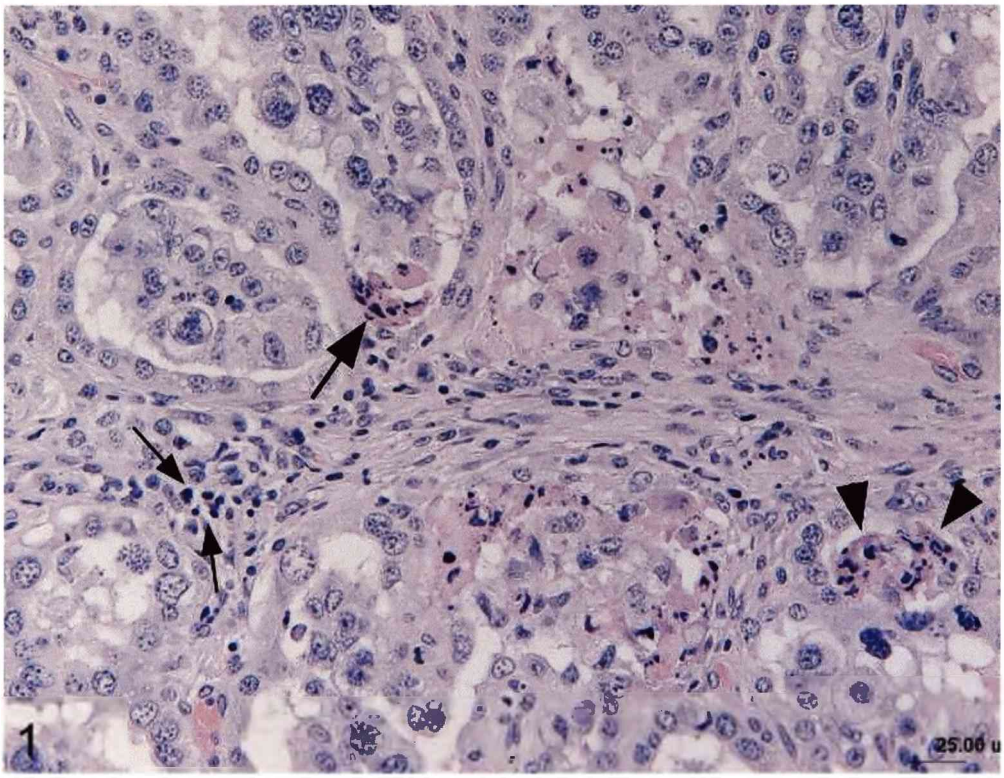



**Figure. 3.4.13.** Placenta after challenge with *Neospora caninum* at 70 dg when foetal death had occurred at day 26 p.i. Widespread epithelial necrosis is evident, affecting both maternal endometrial epithelium (large arrow) and foetal chorionic epithelium (arrow heads). A lymphocyte-dominated mononuclear infiltration is observed in the maternal interstitium (small arrows). HE stain.

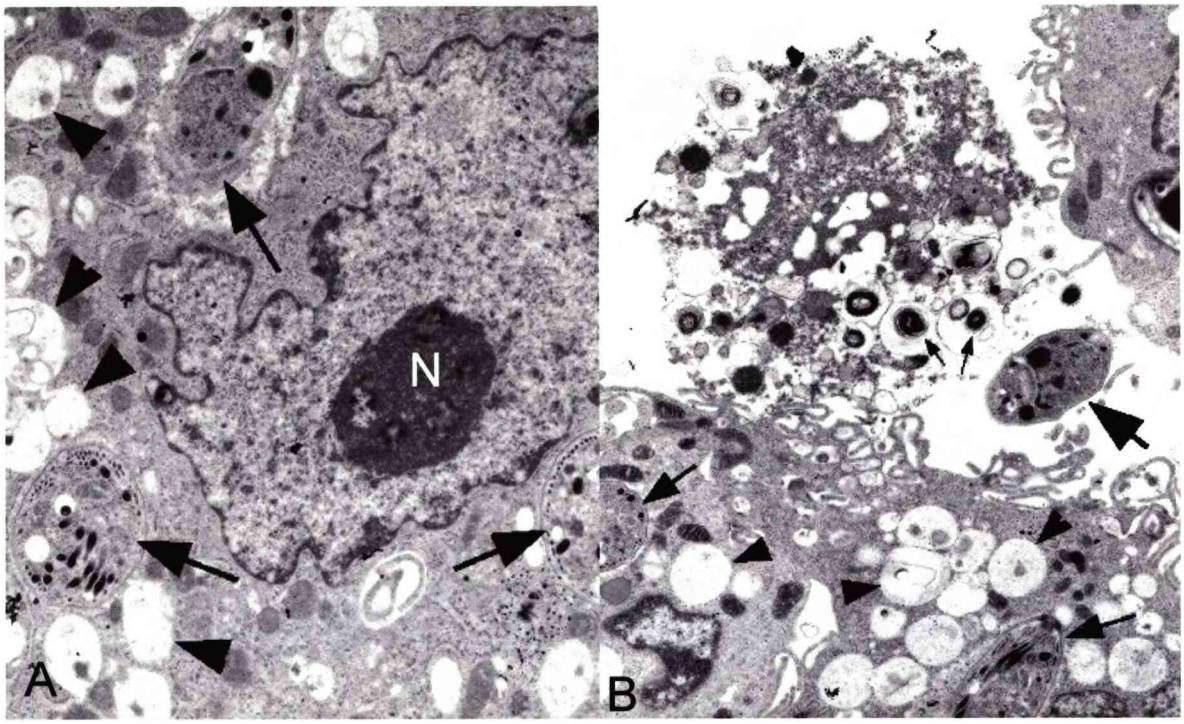



**Figure. 3.4.14**. Transmission electron micrographs of a focal area of necrosis in the placenta after challenge with *N. caninum* at 70 dg when foetal death had occurred at day 19 p.i. (A) Parasite infected maternal epithelial cell. Three tachyzoites (arrows) are evident in the cytoplasm. The cell is exhibiting increased intracytoplasmic vacuolation (arrow heads) and mild nuclear protein margination (N = nucleus). (B) Two tachyzoites (arrows) are present in adjacent epithelial cells that exhibit increased intracytoplasmic vacuolation (arrow heads). A third tachyzoite is cell free (large arrow) adjacent to a cell that has undergone lysis and fragmentation (necrosis). Moderate sphingomyelin (small arrows) figure deposition (remnants of phagolysosomes) is evident.

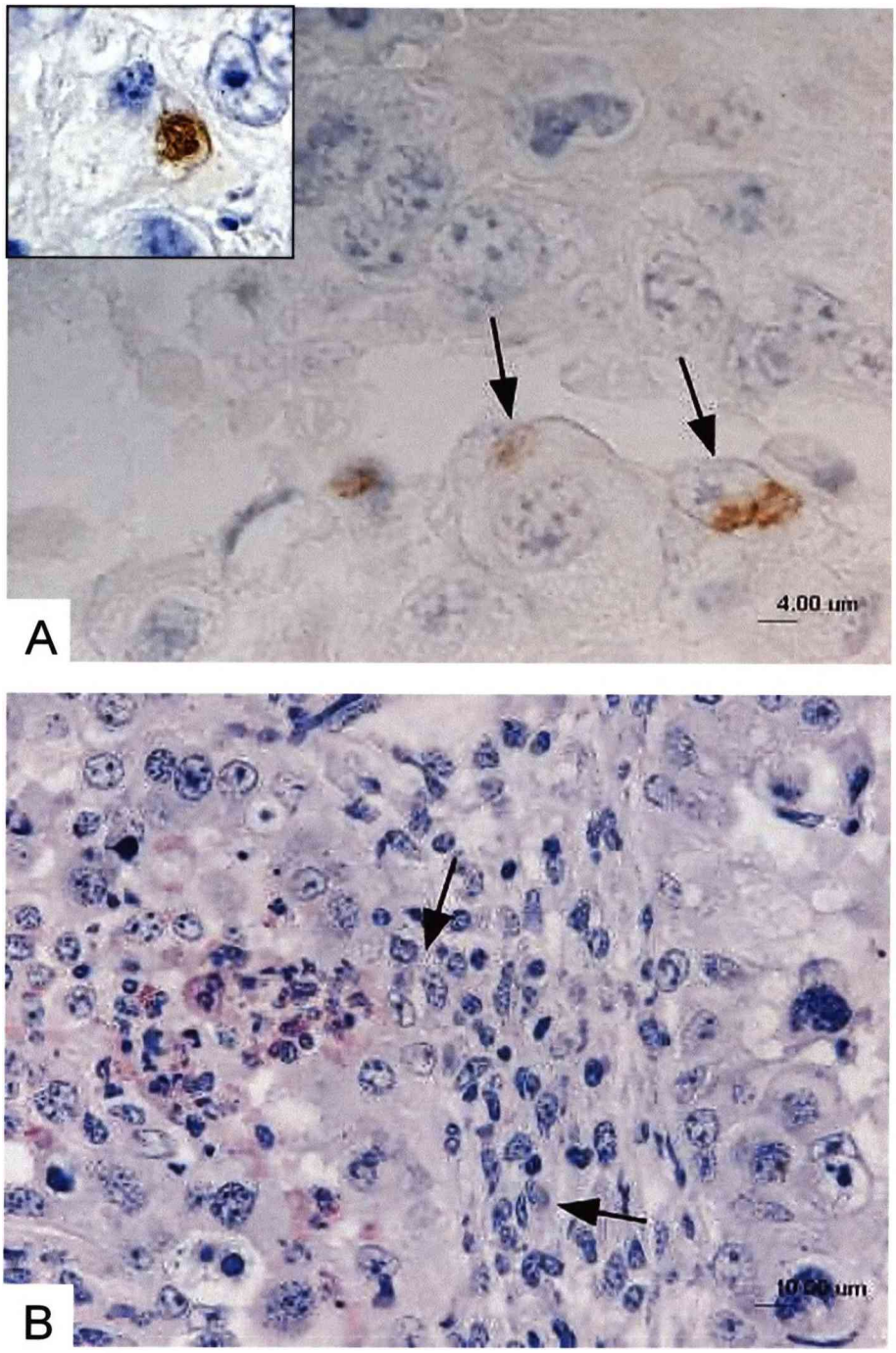



**Figure 3.4.15.** Placenta after challenge with *Neospora caninum* at 70 dg when foetal death had occurred at day 26 (A-C) or 19 (D) p.i. (A) Immunohistology for *N. caninum* antigen identifies tachyzoites in foetal trophoblasts (arrows). Inset: A cluster of tachyzoites is seen within a parasitophorous vacuole in the cytoplasm of an intact foetal trophoblast. PAP method, Papanicolaou's haematoxylin counterstain. (B) Macrophages are found associated with areas of necrosis and in the maternal tissue adjacent to these (arrows). HE stain.

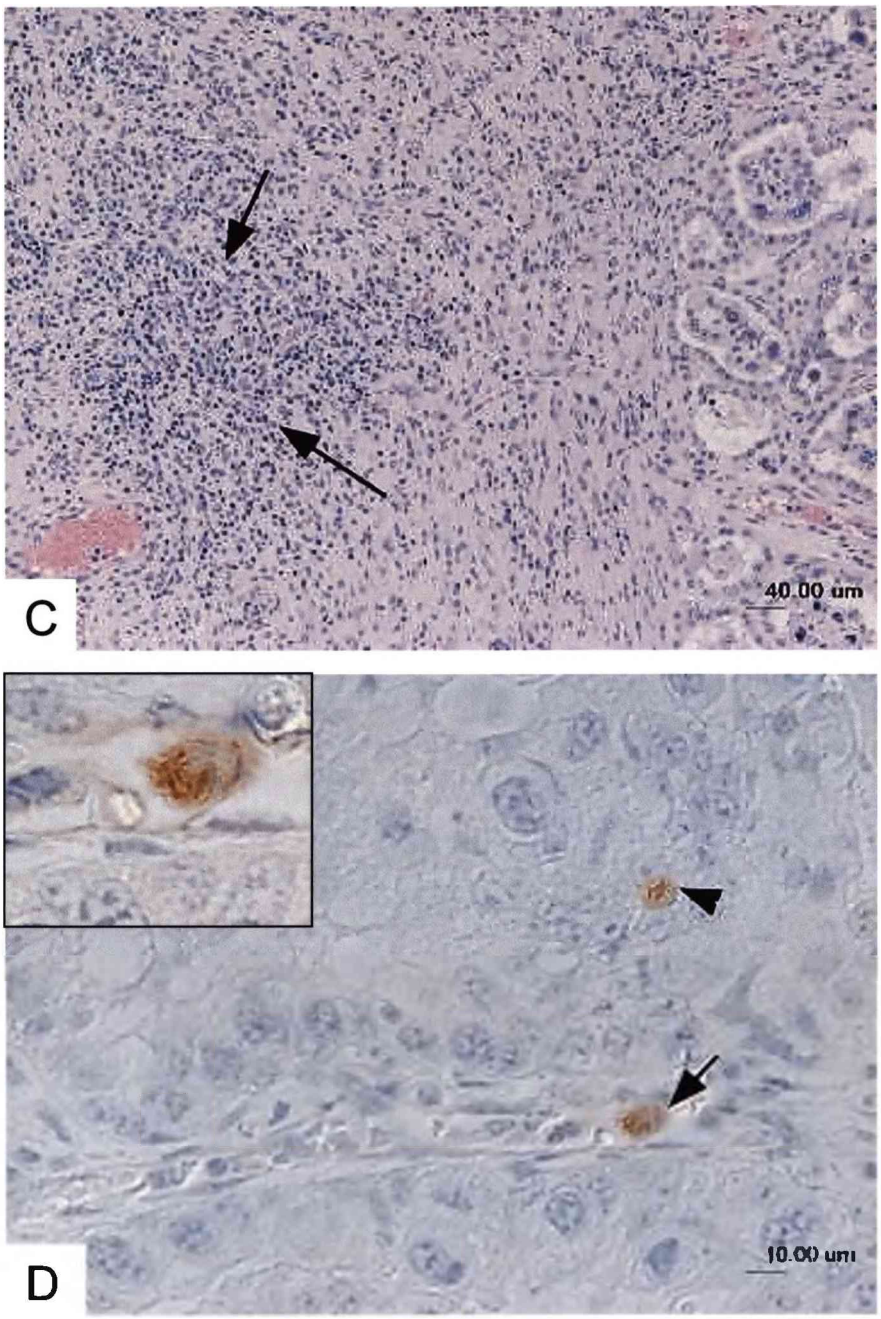



**Figure 3.4.15.** Placenta after challenge with *Neospora caninum* at 70 dg when foetal death had occurred at day 26 (A-C) or 19 (D) p.i. (C) At the base of the caruncles, lymphocyte-dominated perivascular infiltrates are observed (arrows). HE stain. (D) Blood vessel in the maternal placenta containing a maternal leucocyte with four tachyzoites in the cytoplasm (arrow; inset: higher magnification). In addition, a maternal epithelial cell containing tachyzoites is also present (arrowhead). Immunohistology for *N. caninum* antigen, PAP method, Papanicolaou's haematoxylin counterstain.

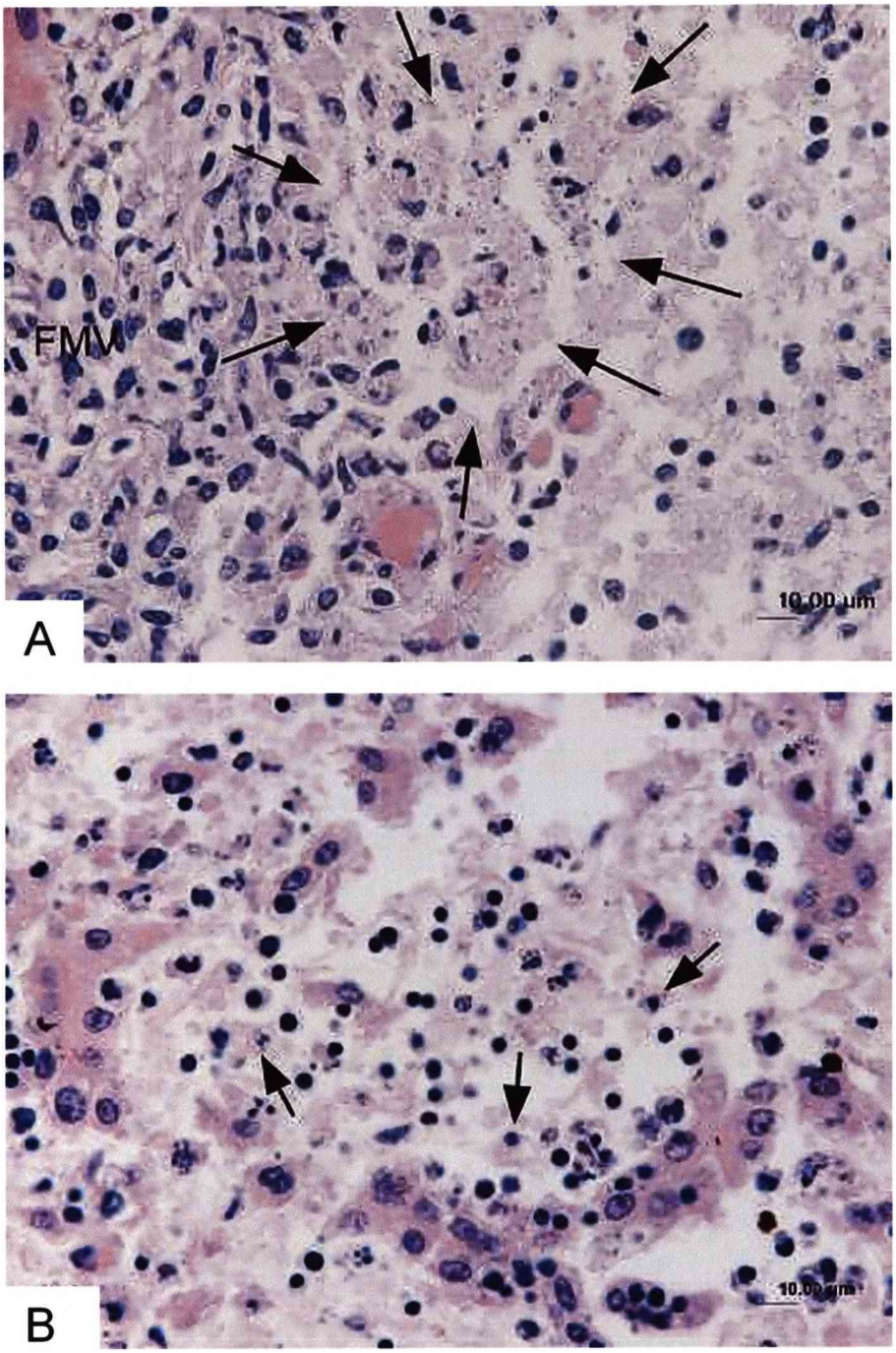



**Figure 3.4.16.** Foetus after challenge with *Neospora caninum* at 70 dg when foetal death had occurred at day 26 (A-D, F) or day 19 (E) p.i. (A) Spinal cord. Focal white matter necrosis (arrows). HE stain. (B) Liver. Multifocal hepatocellular necrosis (arrows). HE stain.

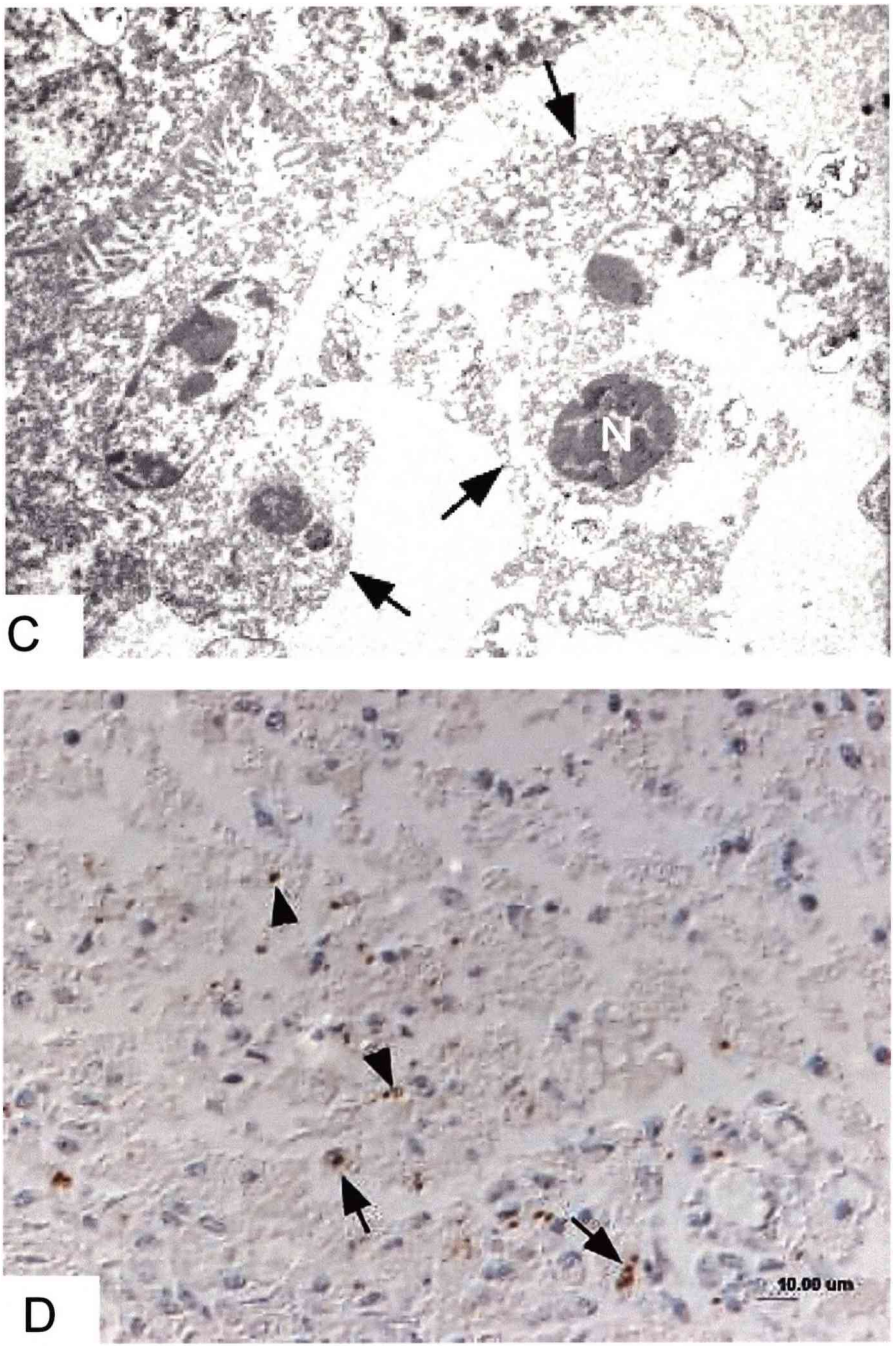



**Figure 3.4.16.** Foetus after challenge with *Neospora caninum* at 70 dg when foetal death had occurred at day 26 (A-D, F) or day 19 (E) p.i. (C) Transmission electron micrograph of necrotic hepatocyte. Arrows point out an indistinct cell border and the loss of cytoplasmic and nuclear membranes. Karyorrhexis of the nucleus (N) is also evident. (D) Spinal cord. Immunohistology for *N. caninum* antigen identifies scattered tachyzoites within glial cells (arrows) and cell free (arrowheads) within the area of necrosis. PAP method, Papanicolaou's haematoxylin counterstain.

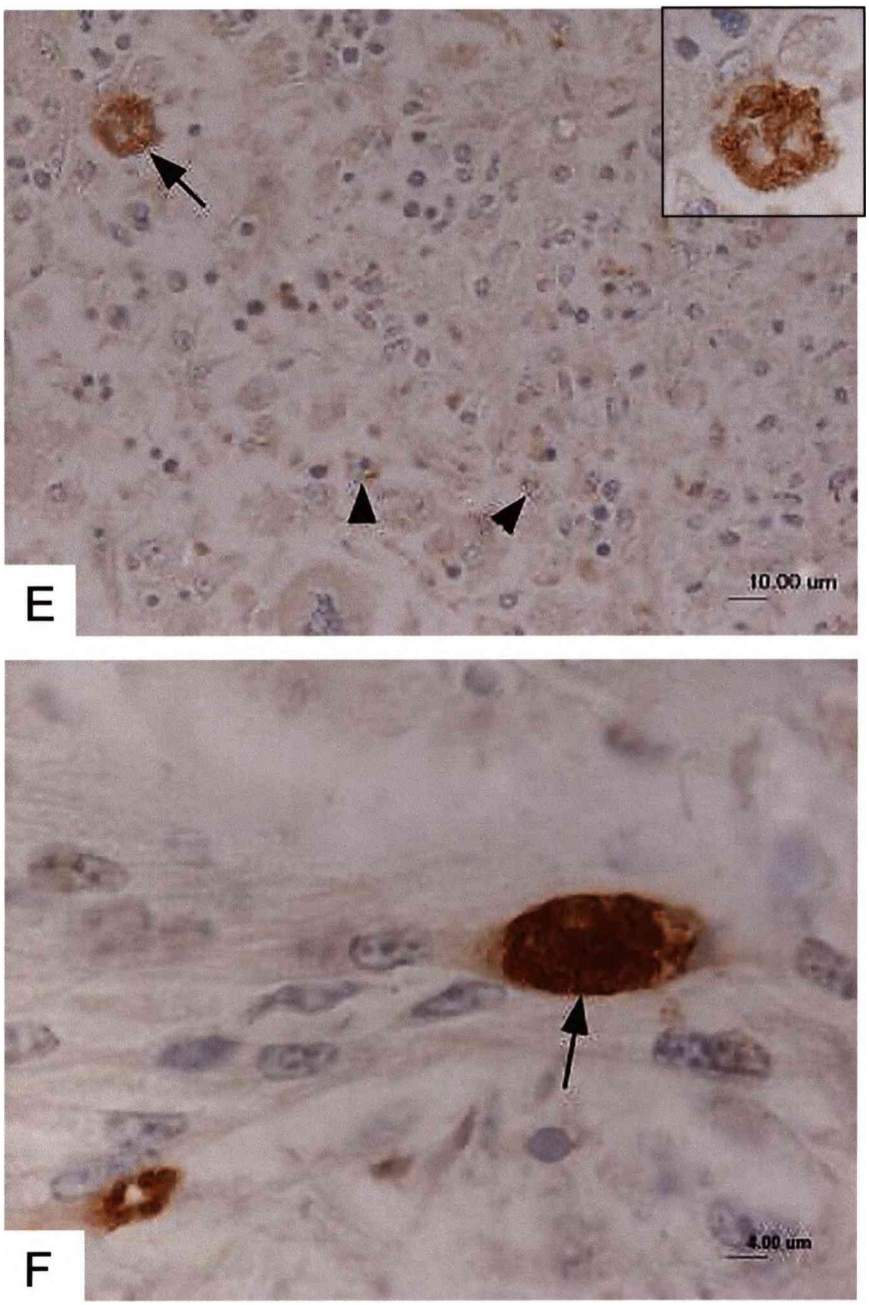



**Figure 3.4.16.** Foetus after challenge with *Neospora caninum* at 70 dg when foetal death had occurred at day 26 (A-D, F) or day 19 (E) p.i. (E) Liver. Immunohistology for *N. caninum* antigen identifies tachyzoites (arrows) within the cytoplasm of a megakaryocyte (inset: higher magnification) and within hepatocytes (arrowheads). PAP method, Papanicolaou's haematoxylin counterstain.(F) Heart. Cluster of tachyzoites within an intact cardiac myocyte (arrow). Immunohistology for *N. caninum* antigen, PAP method, Papanicolaou's haematoxylin counterstain.

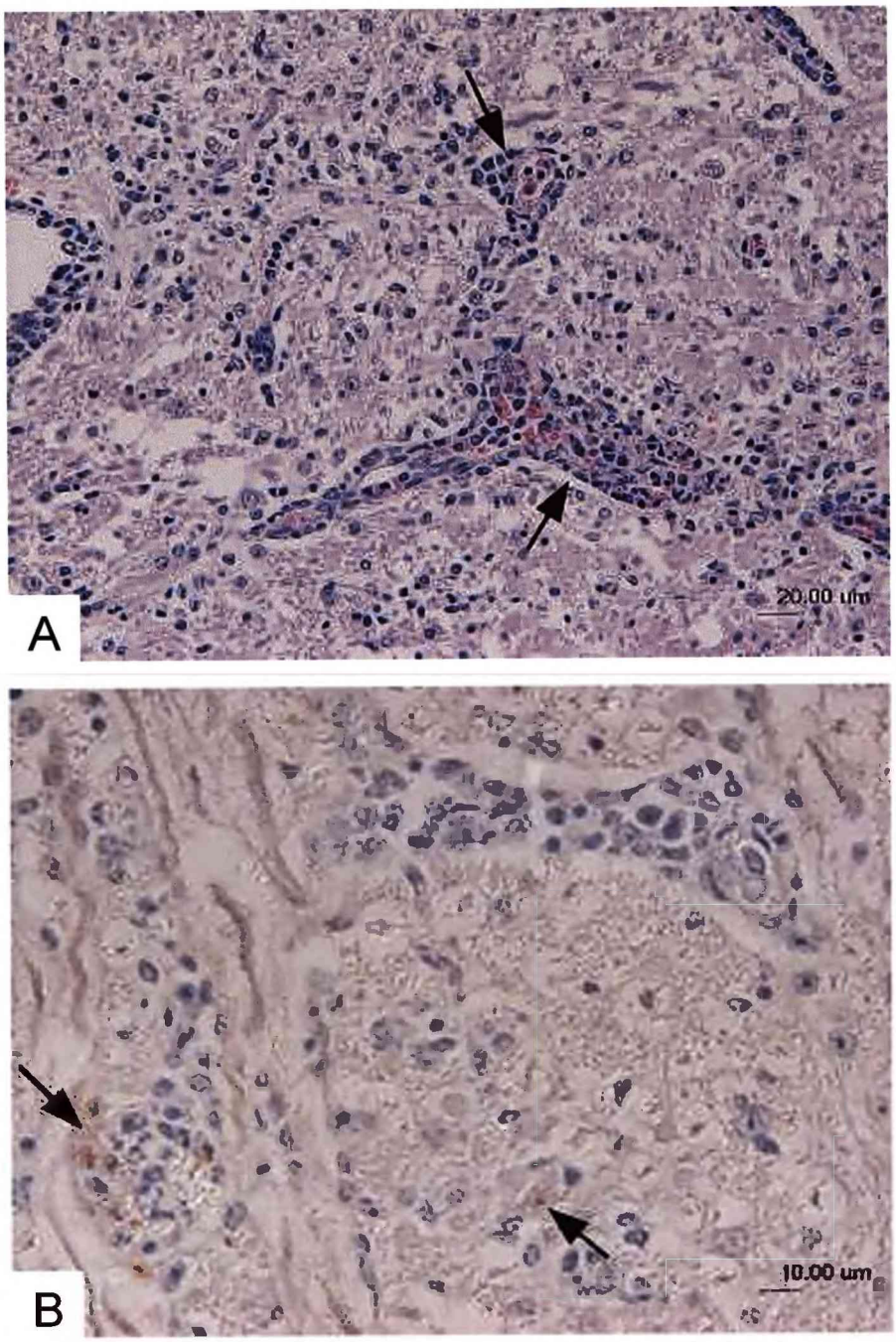



**Figure 3.4.17**. Foetus 21 days after challenge with *Neospora caninum* at 210 dg when foetus was alive on morning of euthanasia. (A) Spinal cord. Focal microgliosis and perivascular mononuclear cell (lymphocytes and macrophages) infiltration (arrows). HE stain. (B) Spinal cord. Immunohistology for *N. caninum* antigen identifies scattered tachyzoites (arrows) within the area of gliosis. PAP method, Papanicolaou's haematoxylin counterstain.

### 3.4.5 Parasite DNA is widespread in placenta and tissues of foetuses that died as a result of infection but found only occasionally in the placenta and foetuses of cattle infected at 210 dg.

Three samples each of placentome and interplacentome area were examined from the six animals infected at 70 dg. *N. caninum* DNA was detected in 17/18 of the placentomes and 7/18 of the interplacentome areas. Brain, spinal cord, lung, kidney and skeletal muscle were positive for *N. caninum* DNA in 7/7 foetuses, the liver in 6/7 and the myocardium in 5/7 foetuses. *N. caninum* DNA was detected in amniotic and allantoic fluid in 4/7 and 1/7 foetuses respectively (Table 3.4.2).

In contrast, after infection at 210 dg, *N. caninum* DNA was only detected in 1/18 of the placentomes and 1/18 of the interplacentome areas. *N. caninum* DNA was detected in one tissue sample only from four of the six foetuses. The positive tissues were brain (1/6), skeletal muscle (1/6) and lung (2/6). *N. caninum* DNA was not detected in the remaining two foetuses and not in the amniotic and allantoic fluid of any of the foetuses (Table 3.4.2).

**Table 3.4.2** Comparison of the detection of parasite DNA in the placenta and foetal tissues from six animals after experimental infection with *N. caninum*, when foetal death occurs in seven foetuses (70 dg) and when the six foetuses survive (210 dg). Three samples of placentome were examined from each of the six animals. Expressed as number of positive samples/number examined.

| **Tissue** | **70 dg[a]** | **210 dg[a]** |
|---|---|---|
| Placentome | 17/18 | 1/18 |
| Interplacentome | 7/18 | 1/18 |
| Brain | 7/7 | 1/6 |
| Spinal cord | 7/7 | 0/6 |
| Liver | 6/7 | 0/6 |
| Heart | 5/7 | 0/6 |
| Lung | 7/7 | 2/6 |
| Kidney | 7/7 | 0/6 |
| Skeletal muscle | 7/7 | 1/6 |
| Allantoic fluid | 4/7 | 0/6 |
| Amniotic fluid | 1/7 | 0/6 |

[a] dg – day of gestation that $10^7$ *N. caninum* tachyzoites were inoculated.

### 3.4.6 *N. caninum* infection at day 70 results in a placental infiltration of CD4+ T lymphocytes and macrophages.

To determine whether the observed mononuclear cell infiltration was associated with recruitment of particular cell types to the placenta, immunohistological staining for T cells, B cells and monocytes/macrophages was performed. In control animals, occasional T and B cells were present, and in the day 210 animals myeloid/histiocyte antigen+ macrophages were scattered in the maternal interstitium. After infection at day 70 of gestation, multifocal villous necrosis was present in all placentomes (Fig 3.4.18A). The areas of epithelial necrosis contained aggregates of myeloid/histiocyte antigen+ cells, which were morphologically identified as macrophages, and macrophages were also numerous around blood vessels at the base of caruncles and in moderate numbers throughout the interstitium (Fig 3.4.18B). There was an intense diffuse lymphocyte infiltration more pronounced at the base of the caruncles, which were CD3+ (Fig 3.4.18C), and consisting of mainly CD4+ cells (Fig 3.4.18D). After infection at day 210, small areas of necrosis were present, but at a much lower frequency than after infection at day 70; these areas contained aggregates of myeloid/histiocyte antigen+ macrophages. A much smaller infiltrate of CD3+ cells which were a mixture of CD4+ and CD8+ T-cells were present mainly around areas of epithelial necrosis. Sparsely scattered CD79a+ B cells were present in placentae collected at both time-points and there were no differences between numbers in control and infected animals.

### 3.4.7 *N. caninum* infection at 70 dg results in expression of IFN-γ in necrotic areas

Expression of IFN-γ was detected in maternal epithelial cells, cell-free within necrotic areas, and in mononuclear cells adjacent to areas of necrosis (Fig 3. 4.18E, F).

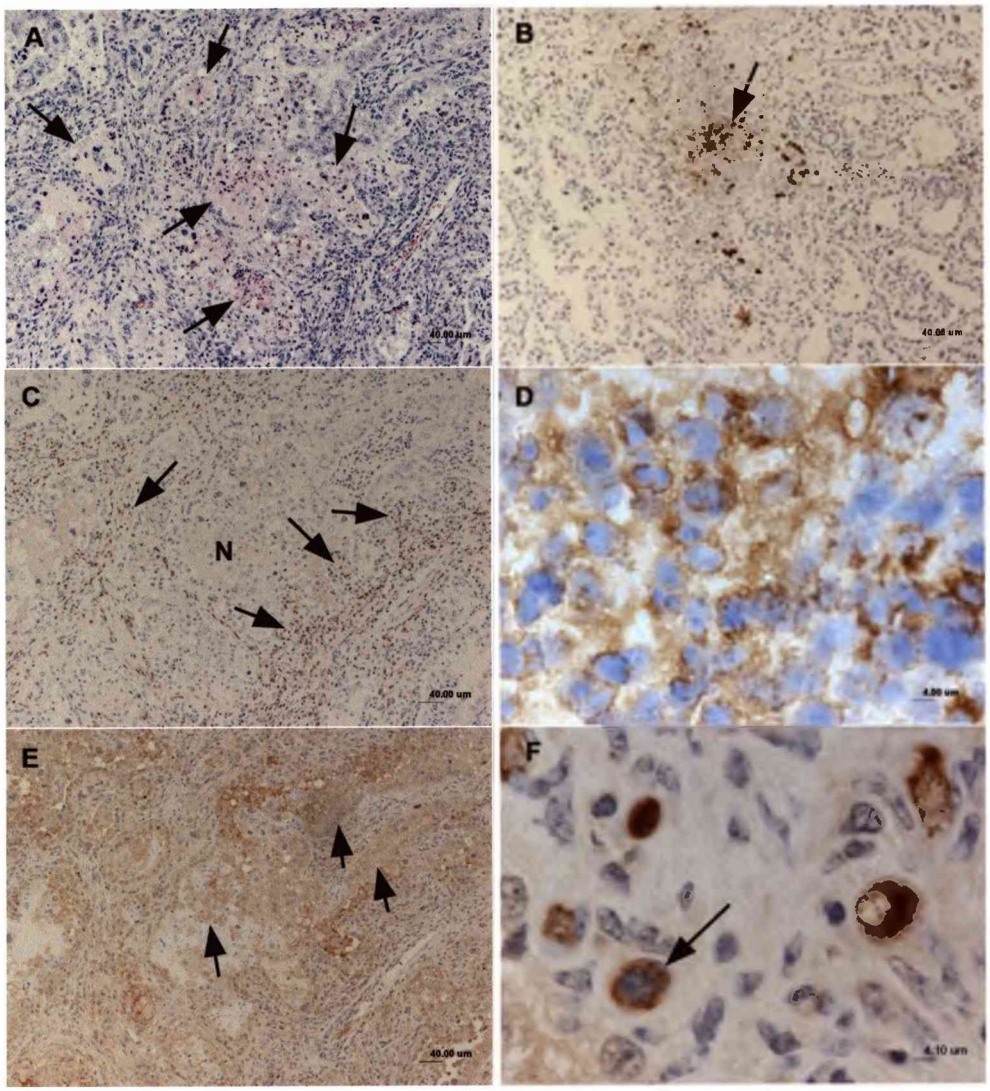



**Figure 3.4.18.** The bovine placenta after challenge with *N. caninum* at day 70 of gestation induces foetal death at day 26 p.i. (A) Multiple small areas of villus epithelial cell necrosis (arrows), surrounded by a mononuclear cell infiltrate in the maternal interstitium. Haematoxylin and Eosin stain. (B) Myeloid/histiocyte antigen + macrophages are dominant cells in association with focal necrosis (arrows). (C) The majority of infiltrating cells in the maternal interstitium are CD3+ T cells (arrows) N: focal area of necrosis. (D) The majority of infiltrating lymphocytes are CD4+. (E) IFN-γ is expressed weakly by maternal epithelial cells (short arrows) and is seen cell-free (arrow) in areas of necrosis. (F) Individual mononuclear cells (arrow: lymphocyte) in the maternal interstitium adjacent to areas of necrosis express IFN-γ. B-F immunohistology using the peroxidase anti-peroxidase method, Papanicolaou's haematoxylin counterstain.

### 3.5 Discussion

The present study compares the pathogenic effect of intravenous infection of pregnant cows with *N. caninum* at an early (70 dg) and late (210 dg) stage of pregnancy, by assessing the histological and ultrastructural changes in placenta and foetal tissues, the inflammatory response and the distribution of the parasite in tissues and cells.

The immune response data showed that all infected animals had antigen-specific antibody production, proliferation of PBMCs and production of IFNγ by PBMCs after infection. There was a significant difference in the PP of infected animals from the two ages groups three weeks after infection, with the mean PP of animals after infection at 210 dg being lower that the mean PP after infection at 70 dg. This is contrary to previous findings, where the two age groups infected showed comparable immune responses (Williams et al., 2000). This could be explained by the fact that the day 210 group all had lower PP values that the day 70 group prior to infection and therefore the rise in PP, although not as great as after infection in the day 70 group, could still be considered significant. Because of this difference in PP values at 3 weeks after infection, and the fact that the infections took place at different time-points with different batches of parasites, we cannot completely rule out the possibility that differences in pathological changes and parasite distribution could be due to differences in infection. However, as stated earlier, previous data would argue against this, as responses previously have been comparable (Williams et al., 2000).

Experimental intravenous inoculation resulted in placental and foetal infection regardless of the stage of gestation when the infection was administered, but there were substantial differences in the extent and pathological effect of infection. After infection at 70 dg, extensive, disseminated placental necrosis with the presence of *N. caninum* tachyzoites both extracellularly and within degenerating/necrotic maternal and foetal epithelial cells were seen, with a macrophage-dominated inflammatory response. Tachyzoites were also seen in a leucocyte within a placental maternal vessel, which to our knowledge demonstrates for the first time that tachyzoites reach the placenta via the maternal circulation. Additionally, transmission electron microscopy demonstrated parasites within the cytoplasm of cells undergoing necrosis, and provides the first *in vivo* evidence of *N. caninum* directly causing necrosis of parenchymal cells.

In comparison, after infection at 210 dg, only very mild focal necrosis with evidence of *N. caninum* infection solely based on the sporadic presence of parasite DNA in the placenta was seen. Nonetheless, after infection at both 70 dg and 210 dg, the placenta exhibited a mild or moderate T cell-dominated mononuclear interstitial infiltration, indicating a general maternal immune response to the agent. The majority of these infiltrating T cells were shown to be CD4+ lymphocytes, which produced IFN-γ.

The maternal interstitial infiltration and widespread necrosis seen in the placenta following infection at d70 and therefore resulting in foetal death is consistent with previous findings, both in field cases (Barr et al.,1990; Dubey and Lindsey,

1996) and experimentally infected cows (Macaldowie et al., 2004). Similarly, immunohistological demonstration of *N. caninum* antigen associated with placental lesions accords with previous studies (Macaldowie et al., 2004).

After infection at 70 dg, all foetuses died 19-26 days after infection. There was extensive necrosis of parenchymal cells in various foetal tissues (brain, spinal cord, liver, kidney, skeletal muscle, lung, pancreas and haemolymphatic tissues), and tachyzoites were seen within necrotic cells. Cardiac myocytes seemed to undergo infection without immediate cell death. Tissue necrosis was not associated with inflammatory infiltrates in the foetus. The dead foetuses also showed evidence of circulating parasitaemia, as parasites were visualised in megakaryocytes in the liver and blood precursor cells in the bone marrow.

The same dose of parasites inoculated at 210 dg did not result in any foetal death, which is consistent with our previous study (Williams et al., 2000). However, in 5/6 foetuses infection was confirmed in foetal tissues either by PCR or immunohistologically, supporting the fact that the parasite must have crossed the placenta into the foetus. Pathological changes were restricted to mild focal encephalitis and myelitis with presence of parasite antigen. This type of lesion has also been reported in natural *Neospora*-associated abortions (Wouda, 2000). Considering that in our study the 210 dg foetuses were alive on the morning of euthanasia and that results of a previous study suggest that these foetuses would most likely have survived to term (Williams et al., 2000), it is possible that the foetal immune response limited the extent of damage the parasite induced at this stage of gestation. It has been shown that the foetal *N. caninum* seroprevalence

increases with gestational age (Barr et al., 1995). Additionally, Andrianarivo et al. (2001) demonstrated strong *Neospora*-specific IgG1 responses along with antigen-specific cell mediated immune responses in foetuses at 219-231 days of gestation.

The differences in the outcome of maternal *N. caninum* infection in the foetus observed in our study suggest that infection early in gestation meets an immunologically immature foetus in which uncontrolled parasitaemia can develop and in which a wide range of parenchymal cell types can become infected by the parasite. The distribution of parasites in the foetus is in agreement with the recent study on natural *Neospora*-associated abortion by Collantes-Fernández et al., (2006), where parasite loads in the brain, heart, kidney and lung by real-time PCR were greatest in foetuses infected in the first trimester.

The inoculation of *N. caninum* tachyzoites at two different stages in gestation resulted in infiltration of lymphocytes in the maternal interstitium and a foetal infection in both cases, but to very different degrees. Late in gestation, both placental inflammation and the numbers of parasites detected in the placenta were restricted, which may have contributed to the foetus surviving challenge. Early in gestation, when foetal death occurred, placental inflammation and numbers of parasites in the foetus were greater, supporting both the theory that foetal death could be due to immune-mediated failure of pregnancy (Innes et al., 2005) and that foetal death could be due to uncontrolled parasitaemia in an immunologically immature foetus.

In this experiment the foetuses of all six animals inoculated at 70 dg were killed. In previous experiments, foetuses have occasionally survived challenge at this stage in gestation. In the one surviving foetus from our previous study (Williams et al., 2000), the foetus survived to term, was serum antibody negative and had no detectable *N. caninum* DNA in the brain when necropsied. Macaldowie et al. (2004) were also unable to demonstrate that foetal infection had occurred in the foetuses that survived experimental infection at 70 dg. From this, it could be suggested that it is the establishment of foetal infection at a time when the foetus is unable to respond to pathogens that is key to determining the outcome of infection. A maternal immune response in the placenta could actually be protective rather than detrimental if it prevents foetal infection.

Differences in the severity of necrosis and inflammation in the placenta could be explained by the differences in the numbers of parasites in the placenta and this could be determined by the level of immunocompetence of the foetus. It is clear from the results presented here that in the younger foetus, parasite multiplication is extensive, resulting in a greater number of parasites than in the older foetuses. These disseminating parasites could then re-invade the placenta in much greater numbers than from the maternal circulation, resulting in extensive necrosis and the greater maternal mononuclear cell infiltration seen in the placenta at this stage in gestation.

Pinpointing which of this sequence of events is responsible for the death of the foetus is difficult. Whilst widespread parasite-associated necrosis of foetal tissues was evident, this may not, depending on extent, lead to foetal death. Equally,

large areas of the placenta were damaged which would lead to placental insufficiency, similar to that seen in toxoplasmosis where placental damage is considered to be the cause of foetal hypoxia and therefore death of the foetus (Buxton et al., 1982). Finally, the placentitis evident in these foetuses could be a further contributing factor.

The majority of infiltrating cells in the placenta after infection early in gestation are CD4+ lymphocytes. After infection early in gestation, proinflammatory (IL-12, IL-18 and IFN-γ) cytokines are expressed at higher levels than late in gestation (Rosbottom et al., 2008). Additionally, we were able to detect IFN-γ in the epithelium and leucocytes of the placenta after infection early in gestation only. It is possible that the Th1 type cytokines produced could play a role in death of the foetus by toxicity to foetal trophoblast cells. However, they are also expressed, albeit at lower levels, late in gestation, therefore whilst their presence is not foetopathic, the differences in level of expression may be significant. The greater number of parasites and necrosis eliciting a stronger inflammatory response could explain this difference.

In summary, all foetuses died following infection early in gestation, and this was associated with widespread placental and foetal necrosis, and widespread parasite dissemination in comparison to the foetuses surviving infection late in gestation. However, in both groups, an active maternal immune response in the placenta was evident by the recruitment of predominantly CD4+ T cells from the circulation and the expression of IFN-γ (Rosbottom et al., 2008). From this it could be suggested that the greater pro-inflammatory immune response early in gestation could be pathogenic. However, in this study we have highlighted the

presence of heavier parasite loads and necrosis in the placenta and have therefore suggested that the level of foetal immunocompetence is the key to determining the outcome of infection, by allowing uncontrolled foetal parasite multiplication, followed by parasite reinvasion and extensive damage to the placenta. If, as is suggested from this experiment, foetal death occurs only in a foetus not able to mount sufficient an immune response to prevent massive parasite amplification, it questions the diagnosis of many *Neospora*-associated abortions in the field, which tend to be found later in gestation when the foetus is mounting an immune response to the parasite. Our experimental evidence to date suggests that these foetuses would survive challenge, and this highlights the need for further investigation on the pathogenesis of abortion in naturally infected animals.

# Chapter 4

# Parasite distribution and lesions after recrudescence of chronic *Neospora caninum* infection in cattle during gestation.

**4.1 Abstract**

The last two decades have seen the emergence of the protozoan parasite *Neospora caninum* as the most frequently diagnosed cause of bovine abortion in the UK. Endogenous transplacental transmission in chronically infected cattle is highly efficient and can result in abortion or the birth of a live, healthy but infected calf at full term. The pathogenic processes that lead to the abortion outcome are not fully understood but previous studies have shown that abortion is more likely if recrudescence of the parasite occurs early in gestation. To investigate further the pathogenesis of abortion, we have monitored ten chronically infected cattle throughout gestation, where parasite recrudescence was pinpointed in 9/10 animals via a sharp rise in *N. caninum*-specific antibodies. All foetuses remained alive until the dams were euthanased 1-5 weeks after the antibody rise. Immunohistology and light microscopy were performed on the placenta and foetal tissues to investigate the histopathological changes and distribution of the parasites. In addition, parasite-specific PCR was carried out on the foetal tissues. The placentae exhibited widespread focal necrosis, with sporadic detection of parasites and a mild to moderate lymphocyte dominated maternal interstitial infiltration. Lesions were detected in 7/10 foetuses and included non-suppurative encephalomyelitis, radiculoneuritis, myositis and myocarditis. Parasites were detected by immunohistology only sporadically in 2/10 foetuses. Parasite DNA was detected in 7/10 foetuses and was most commonly detected in the brain. This study highlights the efficiency with which endogenous transplacental transmission occurs in chronically infected animals. It also reveals that the parasite can be detected in the placenta and foetus, in some cases with associated necrosis and/or inflammation, without

causing foetal death and this has important implications for the diagnosis of *Neospora*-associated abortions.

## 4.2 Introduction

The apicomplexan protozoan *Neospora caninum* is the most frequently diagnosed cause of abortion in dairy cattle in the UK (VLA Surveillance Report 2006). Exogenous transplacental infection can be established following the ingestion of sporulated oocysts (Gondim et al., 2004), but in the majority of cases transplacental transmission is endogenous, occurring in cattle harbouring a chronic infection. Transmission of the parasite to the foetus is highly efficient, with up to 95% of calves born live, healthy and infected at full term (Davison et al., 1999). Therefore, abortion is the clinical outcome of infection in only a minority of cases. The precise mechanism of abortion remains unclear but evidence is gathering to support the theory that foetal death occurs predominantly at a time when the foetus has not yet developed an immune system capable of a competent defence against the pathogen (Gibney et al., 2008). In a previous study of chronically infected animals, foetal death occurred in one animal when the parasites recrudesced early in gestation, whereas in the remaining animals the parasites recrudesced later and the foetuses survived (Guy et al., 2001).

Endogenous transplacental spread, resulting from the recrudescence of bradyzoite cysts in the brain, is the principal natural route of transmission of *N. caninum* in cattle. This suggests reactivation of an established persistent infection, possibly triggered by the "down regulation" of cell-mediated immunity that occurs around mid-gestation (Innes et al., 2001; Innes et al., 2002; Innes et al., 2005). However, although *N. caninum* infection is common, and transplacental spread of the parasite is highly efficient, only a small proportion of

infected cattle abort (Trees et al., 1999). Given that in the majority of cases of endogenous transplacental transmission the foetus survives and that the mechanism by which abortion occurs is not known, this presents difficulty in diagnosing *N. caninum* as the primary cause of abortion, since many other infectious and non-infectious agents are abortifacient (Thurmond et al., 1999). Histopathology is used to indicate that *N. caninum* is the cause of abortion and multifocal necrosis and inflammation have been described in the brain, spinal cord, heart, lung and placenta of field cases (Barr et al., 1991; Lindsay et al., 1993). This is usually carried out in conjunction with immunohistological staining for the parasite (Lindsay and Dubey, 1989). The finding of a parasite-associated lesion in the foetus is taken as confirmation that the parasite was the cause of abortion. In recent years, parasite specific PCR has been considered as a means of diagnosis, but this only confirms the presence of the parasite in the foetus rather than demonstrating any pathological lesions and so does not give any indication as to whether the parasite could have been the cause of abortion. Serological assays have also been used in the foetus or dams (reviewed by Björkman and Uggla, 1999).

Controlled studies on naturally, chronically infected animals are scarce. Given that abortion occurs in only a minority of cases of parasite recrudescence in chronically infected animals, there is some confusion as to what lesions or circumstances can be used to definitively diagnose a *Neospora*-associated abortion. The aim of this study was to investigate the pathogenic processes that take place in the placenta and foetus in association with parasite recrudescence in the context of whether or not foetal death occurs. We monitored ten naturally,

chronically infected cattle throughout gestation, and pinpointed when parasite recrudescence occurred via a sharp rise in *N. caninum*-specific antibodies. The dams were euthanased within 1-5 weeks of recrudescence and placental and foetal tissues recovered and used to investigate the histopathological changes and distribution of the parasites in the placenta and foetus.

## 4.3 Materials and Methods

### 4.3.1 Project design and sample collection

#### *4.3.1.1 Source and husbandry of cattle*

10 Holstein Friesian cows aged 2 – 7 years were purchased from commercial dairy farms, and were selected on the basis of having a confirmed *Neospora*-associated abortion in a previous lactation. The cattle were tested to ensure they were persistently infected with *N. caninum* as evident by detection of *N. caninum* specific antibodies above the test positive cut off value of PP 20 (Mastazyme, Mast Diagnostics, UK) and were tested negative for bovine viral diarrhoea virus by the Veterinary Laboratories Agency, Preston, UK. Any potential chronic leptospiral infections were treated with Procaine Penicillin and Dihydrostreptomycin Sulphate (Streptacare® Animalcare Limited, UK) at a dose rate of 20 mg/kg penicillin and 25 mg/kg streptomycin by intramuscular injection daily for three days after purchase. Vaccination was carried out against Leptospira (Leptavoid-H, Schering-Plough, Uxbridge, UK), bovine viral diarrhoea virus (Bovidec, Novartis, Royston, UK) and infectious bovine rhinotracheitis virus (Bovilis IBR marker, Intervet, Milton Keynes, UK).

The cattle were oestrus synchronised using progesterone releasing intra-uterine devises (PRID, CEVA Animal Health, Watford, UK), artificially inseminated with certified disease-free Holstein Friesian semen, and housed with a Hereford bull immediately after insemination. Animals were maintained in dog and fox proof straw-bedded pens at Liverpool University Farm, Leahurst Campus, with free access to hay and water and fed concentrates twice daily. Pregnancy and foetal viability were confirmed at 35 days by trans-rectal ultrasonography. A

Holstein Friesian cow that was not infected with *N. caninum* was used as a sentinel and housed with the group for the duration of the experiment.

*4.3.1.2 Clinical monitoring and collection of blood samples*

Cattle were observed daily throughout the experimental period. Blood samples were collected from all animals by jugular venipuncture into plain evacuated tubes (BD Ltd, Oxford, UK) weekly for the duration of the experiment. Samples were allowed to clot at 4 °C overnight before centrifugation at 1000 g for 10 min and removal of serum, which was then divided into aliquots and stored at −20 °C until required.

Foetal viability was monitored by ultrasonography at weekly intervals, transrectally until month 5 of gestation, and transabdominally thereafter. *N. caninum* specific antibody levels of the cattle were also monitored weekly (see section 4.2.2) and if an increase in antibody level was detected, scanning was increased to three times weekly to monitor foetal viability until euthanasia.

During the project design stages, the decision was made to class parasite recrudescence as a 50% increase in *N. caninum* specific antibody level. This was based on a previous experiment, where a 50% increase in *N. caninum* specific antibodies indicated parasite recrudescence (Guy et al., 2001). In this previous experiment, foetal ultrasonography 6 days after the antibody increase was detected, revealed that foetal death had occurred in one animal following recrudescence at 17 weeks gestation. Additionally, in the experiment detailed in chapter 3, all foetuses infected at 210 days gestation survived. Based on this information, animals were subjected to a post-mortem examination as soon as

possible after a 50% increase in antibody level was detected and remained high a week later. This was to be confident that foetal death was unlikely to occur, but to enable the foetus and placenta to be examined as soon as possible after parasitaemia. Animals where no sharp increase in antibody level was detected during pregnancy, or in which the antibody increase occurred after week 36 of gestation, were left to calve normally and a precolostral blood sample taken from the calves to test for *N. caninum* specific antibodies. Calves were euthanased at approximately two weeks of age.

*4.3.1.3 Euthanasia and collection of tissue samples*

Euthanasia of the adult cattle was carried out using a captive bolt pistol followed by pithing. Immediately after euthanasia, the uterus was removed from each animal and the foetus or foetuses recovered. Ten randomly selected samples of placentome and inter-placentome area were collected from each uterus. Maternal spleen and uterine (sub-iliac) lymph node was also collected. Foetal tissue samples collected included the brain and spinal cord, an apical section of the heart, the lung, the main lobe of the liver, the spleen, the left kidney and adrenal gland, the pancreas, a section of jejunum, the quadriceps skeletal muscle, a femoral nerve, the umbilical cord, bone marrow (BM) from the femur, the thymus, and the mesenteric lymph node (MLN).

For all of these tissues, a sample was collected into 4% buffered paraformaldehyde (pH 7.4, see Appendix) for histology and a further sample collected aseptically and frozen for PCR. In addition, samples of amniotic and allantoic fluid were collected in a sterile manner using a 19 g needle and 2 ml syringe prior to opening the uterus, and frozen for PCR.

Euthanasia of the calves was carried out by intravenous injection of pentobarbitone sodium at a dose rate of 200 mg/kg (Pentoject, Animalcare, UK). Tissue samples collected included the brain and spinal cord, an apical section of the heart, the lung, the main lobe of the liver, the spleen, the left kidney and adrenal gland, the pancreas, a section of jejunum, the quadriceps skeletal muscle, a femoral nerve, the umbilical cord, BM from the femur, the thymus, and the MLN.

### 4.3.2 *N. caninum* antibody levels of cattle during pregnancy

Weekly serum samples from all animals and precolostral serum samples from the calves were tested for *N. caninum*-specific antibodies measured using the Mastazyme serum antibody ELISA, as detailed in section 3.2.2.1. In the pregnant animals, recrudescence of the parasite was indicated by a sudden sharp increase in PP value of 50% or greater.

### 4.3.3 Preparation of samples for histopathology

#### *4.3.3.1 Sectioning of tissues*

After fixation for 5 days, samples were trimmed into cassettes for processing. For each animal, transverse sections of the 10 placentomes were taken to include the attached inter-placentome area and uterine wall. Lymph nodes were sectioned in the transverse plane through the hilus. Foetal brain was sectioned to include transverse sections of frontal cerebrum, occipital cerebrum and hippocampus, midbrain, cerebellum and medulla. Transverse samples were obtained from cervical, thoracic, lumbar and sacral areas of the spinal cord. Representative

portions of all other tissues were examined. After trimming, the samples were processed through graded alcohols and a xylene step, before being embedded in paraffin wax. Sections 3 - 5 μm thick, were stained with haematoxylin and eosin (HE) and examined for any pathological changes or were used for immunohistology.

*4.3.3.2 Haematoxylin and Eosin staining*

Haematoxylin and eosin staining was carried out as described in section 3.2.3.2.

4.3.4 Detection of *N. caninum* by immunohistology

For each section that was examined histologically, a subsequent 3 - 5 μm section was cut, mounted onto a poly-L-lysine coated slide, and examined immunohistologically for *N. caninum*, as described in section 3.2.4.

4.3.5 Detection of *N. caninum* by polymerase chain reaction

From each foetus or calf, a sample of brain, heart and liver was examined for *N. caninum* DNA, as described in section 3.2.5.

## 4.4 Results

### 4.4.1 Parasite recrudescence occurs in all animals during gestation, without causing foetal death, and is associated with a sharp rise in *N. caninum* specific antibody levels in 9/10 animals.

Weekly monitoring of *N. caninum* specific antibody levels throughout gestation revealed a sudden, sharp rise in antibody levels in 9/10 animals, between week 20 and week 37 of gestation (Fig. 4.4.1). Following the rise in antibody levels, foetuses remained alive until the day of euthanasia, 1 – 5 weeks later (Table 4.4.1). Animals 1 and 7 were carrying twin foetuses. In animal 9, the rise in antibody level occurred late in gestation (week 37); this animal was left to calve normally and the calf was euthanased 2 weeks later. In animal 10, there appears to be rising antibody levels at the end of gestation (week 40). This animal was also left to calve normally and the calf euthanased 2 weeks later.

In animals 1-8, where a rise in antibody level was detected between 20 –33 weeks of gestation, parasite recrudescence was confirmed in 8/10 foetuses by either immunohistological demonstration of *N. caninum* in the placenta or foetus, or by the presence of parasite DNA in the foetus. In both animal 9, where a rise in antibody level was detected at week 37 of gestation, and animal 10, where an increase in antibody level was detected at the end of gestation, precolostral blood samples revealed the presence of *N. caninum*-specific antibody in both calves (Table 4.4.2), confirming that parasite recrudescence and endogenous transplacental transmission had occurred in these animals also. The sentinel animal (11) remained uninfected with *N. caninum* for the duration of the experiment.

**Figure 4.4.1: Immune responses in 10 pregnant cattle chronically infected with *N. caninum* and one uninfected animal (11): *N. caninum* specific antibody responses in an ELISA shown as percent positivity (PP) of a positive control serum.**

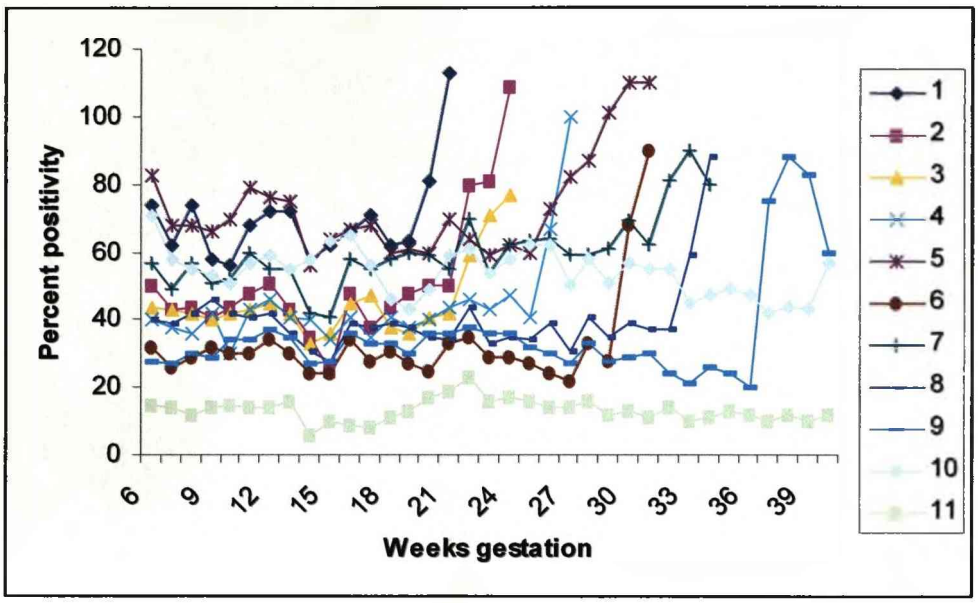


**Table 4.4.1: Timing during gestation of a sharp rise (50% increase) in *N. caninum*-specific antibody levels relative to timing of euthanasia and sample collection for 10 chronically infected cattle.**

| Animal number | First increase in antibody (week of gestation) | Euthanasia (week of gestation) |
|---|---|---|
| 1 | 20 | 21 |
| 2 | 22 | 24 |
| 3 | 22 | 24 |
| 4 | 26 | 27 |
| 5 | 26 - 29 | 31 |
| 6 | 30 | 31 |
| 7 | 32 | 34 |
| 8 | 33 | 34 |
| 9 | 37 | 2 weeks post calving |
| 10 | 40 | 2 weeks post calving |

**Table 4.4.2: Precolostral *N. caninum* antibody levels in 2 calves from chronically infected dams (results expressed as a percent positivity of a high positive control - >20 is positive).**

| Calf number | Percent positivity |
|---|---|
| 9 | 99 |
| 10 | 59 |

**4.4.2 Following recrudescence in chronically infected animals, focal necrosis, with sporadic detection of parasites was detected in the placenta together with a mild to moderate lymphocyte dominated maternal interstitial infiltration.**

Discrete areas of focal epithelial necrosis were identified in the placenta of the eight cows killed during gestation. All placentomes examined (n=10 per animal) exhibited several small areas of epithelial necrosis (Fig. 4.4.2A), generally affecting a single foetal villous (Fig. 4.4.2B). More extensive necrosis affecting the surrounding maternal epithelium was occasionally seen. Larger areas of necrosis affecting several foetal villi and the surrounding maternal epithelium were evident in all of placentomes from animals 5 and 6. In these animals, focal haemorrhage and focal serum leakage was present at the villous tips or in the spaces between the maternal and foetal cell layers (Fig. 4.4.3A).

In addition, in all eight animals a mild, diffuse, lymphocyte-dominated, mononuclear interstitial infiltration was present. In animals 5 and 6, this infiltration was mild to moderate, with greater numbers of lymphocytes seen in the maternal interstitium surrounding necrotic epithelium (Fig. 4.4.3B).

*N. caninum* antigen was demonstrated by immunohistology in the placenta of animals 1, 4 and 6. Staining was most intense in animal 1, where antigen was identified in 6/10 placentomes examined. Antigen was observed both cell free (Fig.4.4.4A) and within degenerating cells in the areas of foetal chorionic villous necrosis (Fig. 4.4.4B). In animals 4 and 6, *N. caninum* antigen was identified in 1/10 placentomes examined and was present within intact maternal endometrial and foetal trophoblast epithelial cells.

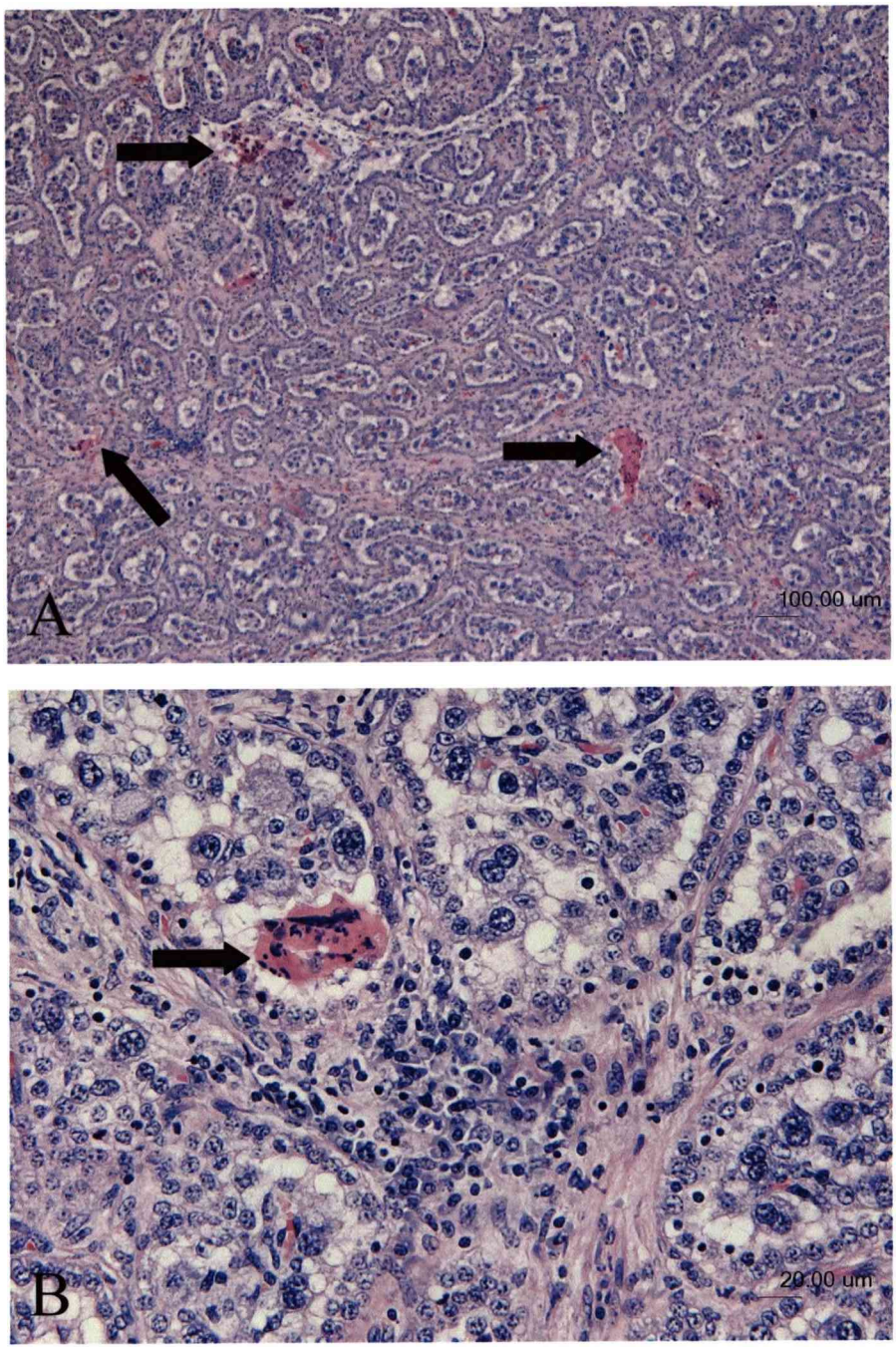



**Figure 4.4.2** Placenta of animal 2 following parasite recrudescence at 22 weeks gestation and necropsy at 24 weeks gestation. (A) Overview of placenta showing several small areas of epithelial necrosis (arrows). (B) Necrosis of a single foetal chorionic villous (arrow). HE stain.

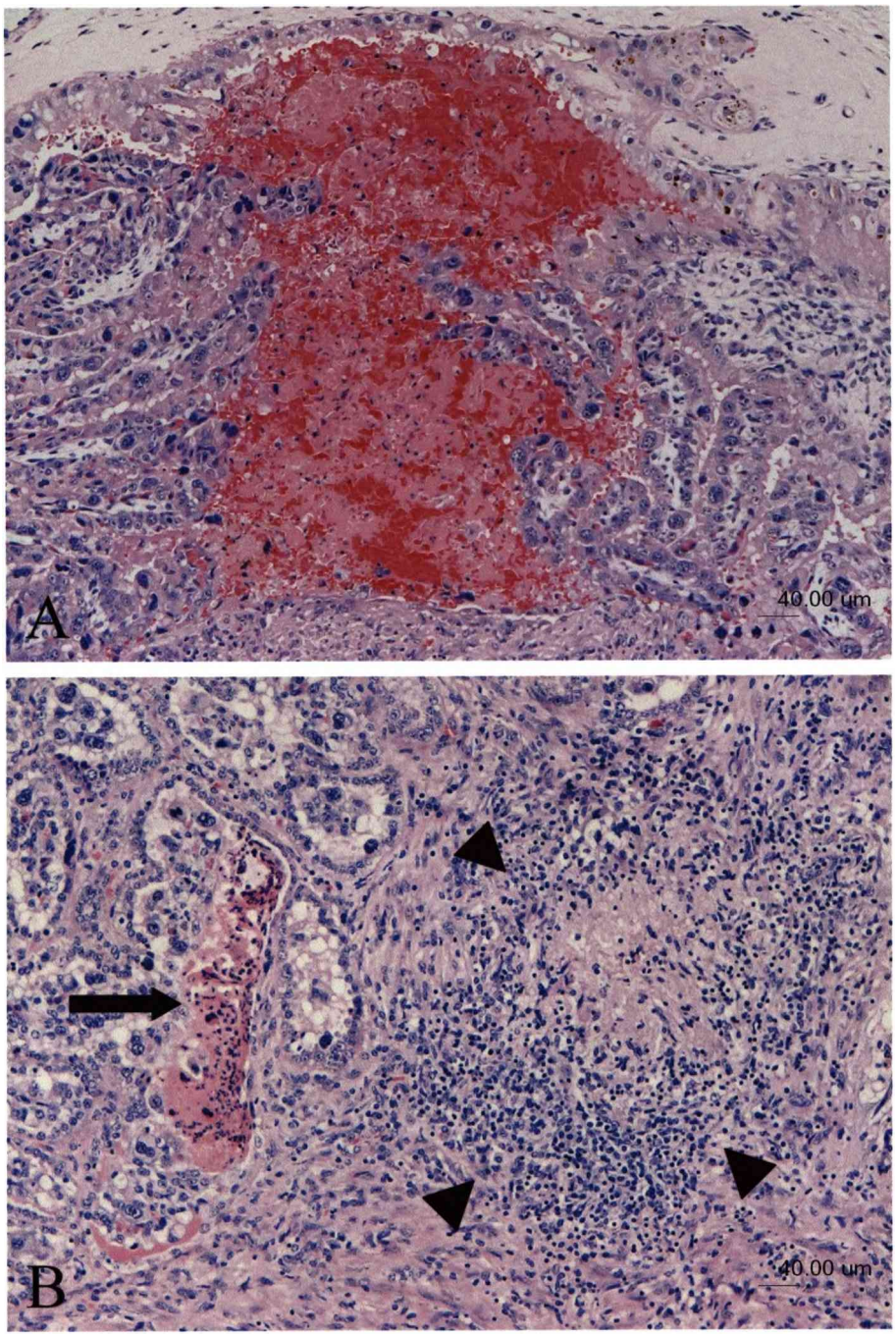



**Figure 4.4.3** Placenta of animal 5 after recrudescence at 26 weeks gestation and necropsy at 31 weeks gestation. (A) Focal haemorrhage and serum leakage is evident at the villous tips. (B) A mild to moderate lymphocyte dominated maternal interstitial infiltration is evident (arrowheads) adjacent to an area of epithelial necrosis (arrow). HE stain.

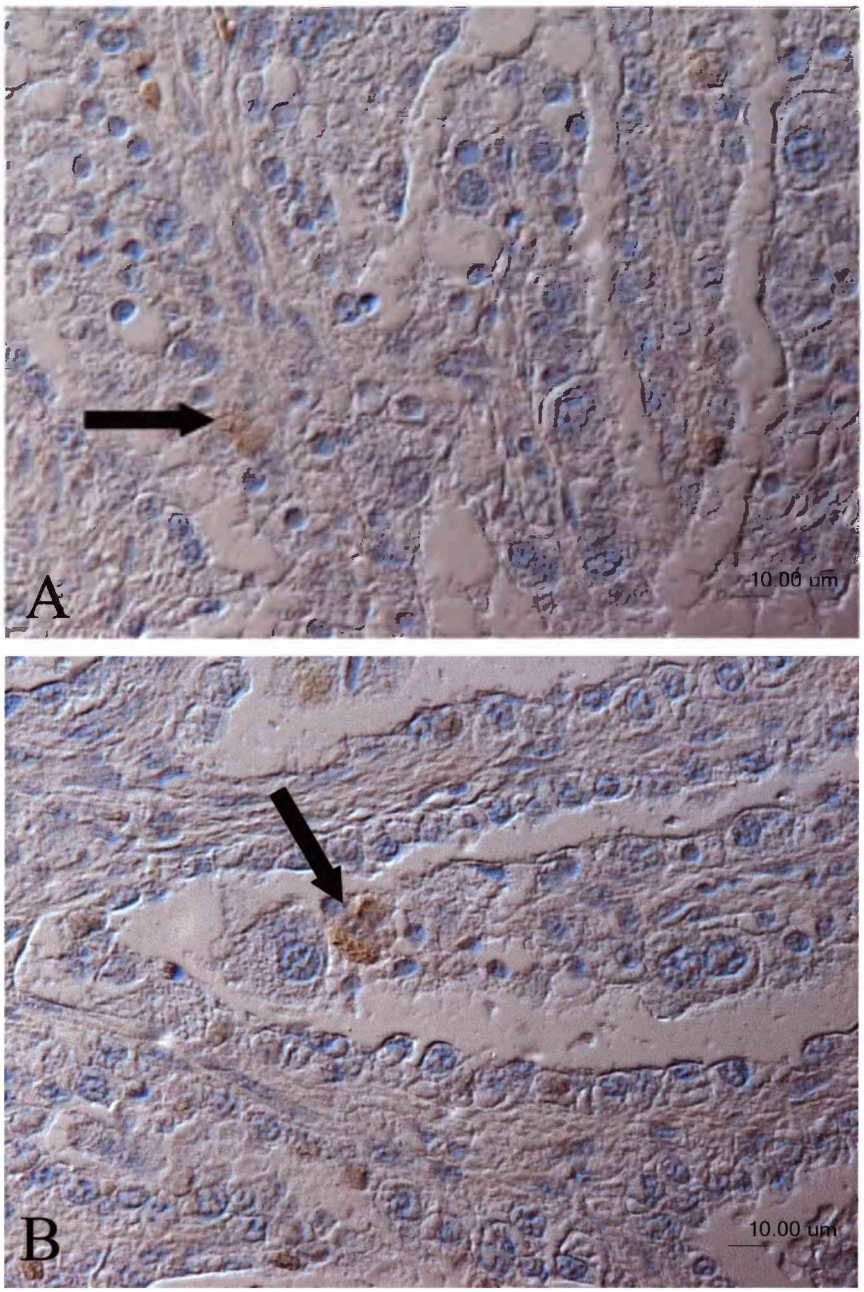



**Figure 4.4.4** Placenta of animal 1 after parasite recrudescence at 20 weeks gestation and necropsy at 21 weeks gestation. Immunohistology for *N. caninum* antigen identifies cell free tachyzoites (A – arrow) and tachyzoites within foetal chorionic epithelial cells (B - arrow) in areas of necrosis. PAP method, Papanicolaou's haematoxylin counterstain.

**4.4.3 Non-suppurative inflammatory lesions are detected in 7/10 of the foetuses examined after parasite recrudescence in chronically infected dams, but parasites are detected only sporadically.**

Histological alterations were detected in 7/10 foetuses and parasite antigen was demonstrated immunohistologically in 2/10 foetuses following parasite recrudescence. In foetus 1a, the histological findings were restricted to a moderate multifocal non-suppurative myositis. Parasite antigen was not detected. Foetus 1b exhibited mild focal non-suppurative myelitis, represented by a focal aggregate of macrophages with fewer lymphocytes and plasma cells, in the white matter and a moderate multifocal non-suppurative myocarditis and myositis, all without presence of parasite antigen. Foetus 2 exhibited focal non-suppurative encephalitis, represented by a focal aggregate of macrophages with fewer lymphocytes and plasma cells and focal mild, lymphocyte-dominated perivascular infiltration in the medulla oblongata, and focal non-suppurative myelitis, represented by a focal aggregate of macrophages with fewer lymphocytes and neutrophils and focal mild, lymphocyte-dominated perivascular infiltration in the white matter. Parasite antigen was not detected in brain and spinal cord. Mild focal, non-suppurative myositis was identified (Fig. 4.4.5A) and parasites were seen within an intact myocyte immediately adjacent to the inflammatory infiltrate (Fig.4.4.5B). Foetus 3 exhibited mild focal non-suppurative myelitis and mild acute, focal mixed cellular (macrophages, neutrophils, a few lymphocytes) myositis with haemorrhage, both again without evidence of parasite antigen. In the spinal cord of foetus 5, moderate multifocal leptomeningeal mononuclear (lymphocytes, plasma cells, macrophages) infiltration and non-suppurative myelitis, represented by focal parenchymal

aggregates and adjacent perivascular infiltrates of lymphocytes, plasma cells and macrophages was evident (Fig. 4.4.6A and B), without evidence of parasite antigen. Severe, multifocal bilateral non-suppurative radiculoneuritis, with presence of parasite antigen was also seen (Fig. 4.4.7A and B). Mild focal non-suppurative myositis, without evidence of parasite antigen, was also present. Foetus 6 exhibited mild focal non-suppurative encephalitis, represented by a focal aggregate of macrophages and lymphocytes in the frontal cortex, myelitis (represented by perivascular, lymphocyte-dominated mononuclear infiltration) and radiculoneuritis. A focal non-suppurative myositis was also observed. Again no parasite antigen was detected. In foetus 8, mild focal non-suppurative encephalitis, represented by a focal aggregate of macrophages, lymphocytes and occasional plasma cells and moderate multifocal non-suppurative myelitis, represented by focal aggregates and adjacent perivascular infiltrates by predominantly macrophages and lymphocytes, and radiculoneuritis as well as mild chronic non-suppurative, mainly interstitial myositis and myocarditis were seen. Parasite antigen was not detected. The remaining foetuses 4, 7a and 7b did not show any significant pathological changes and parasite antigen was not detected by immunohistology.

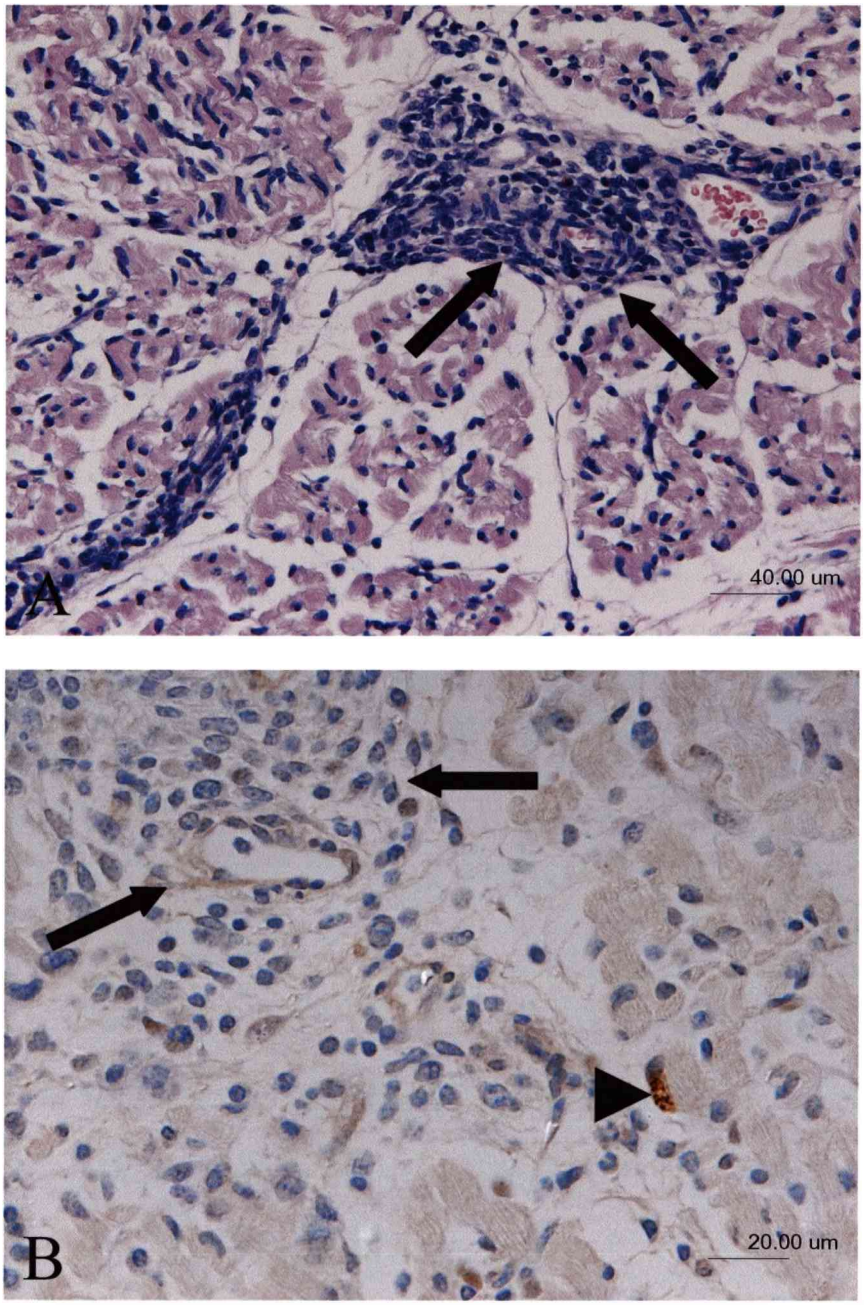



**Figure 4.4.5** Foetus 2 following parasite recrudescence at 22 weeks gestation and necropsy at 24 weeks gestation. The foetus exhibited mild focal, non-suppurative myositis (A - arrows; HE stain). (B) Immunohistology for *N. caninum* antigen identifies tachyzoites within an intact myocyte (arrowhead) immediately adjacent to the inflammatory infiltrate (arrows). PAP method, Papanicolaou's haematoxylin counterstain.

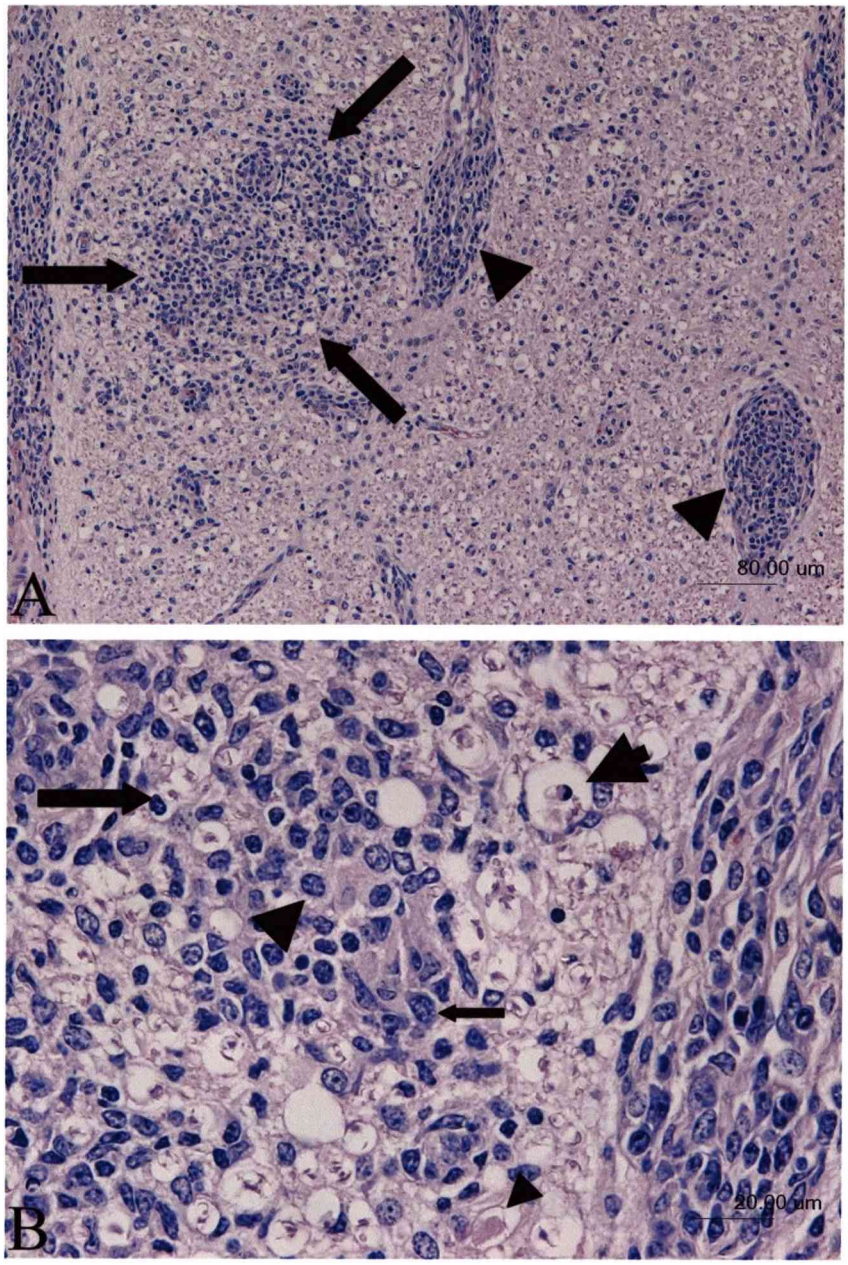



**Figure 4.4.6** Foetus 5 after recrudescence at 26 weeks gestation and necropsy at 31 weeks gestation. A moderate multifocal non-suppurative myelitis was seen. (A) This was represented by focal parenchymal (arrows) and adjacent perivascular (arrowheads) mononuclear infiltration. HE stain. (B) This infiltration consisted of lymphocytes (large arrow), plasma cells (large arrow head) and macrophages (small arrow). In association with the infiltrate, occasional spheroids (small arrowhead) and myelinophages (short arrow) are seen. HE stain.

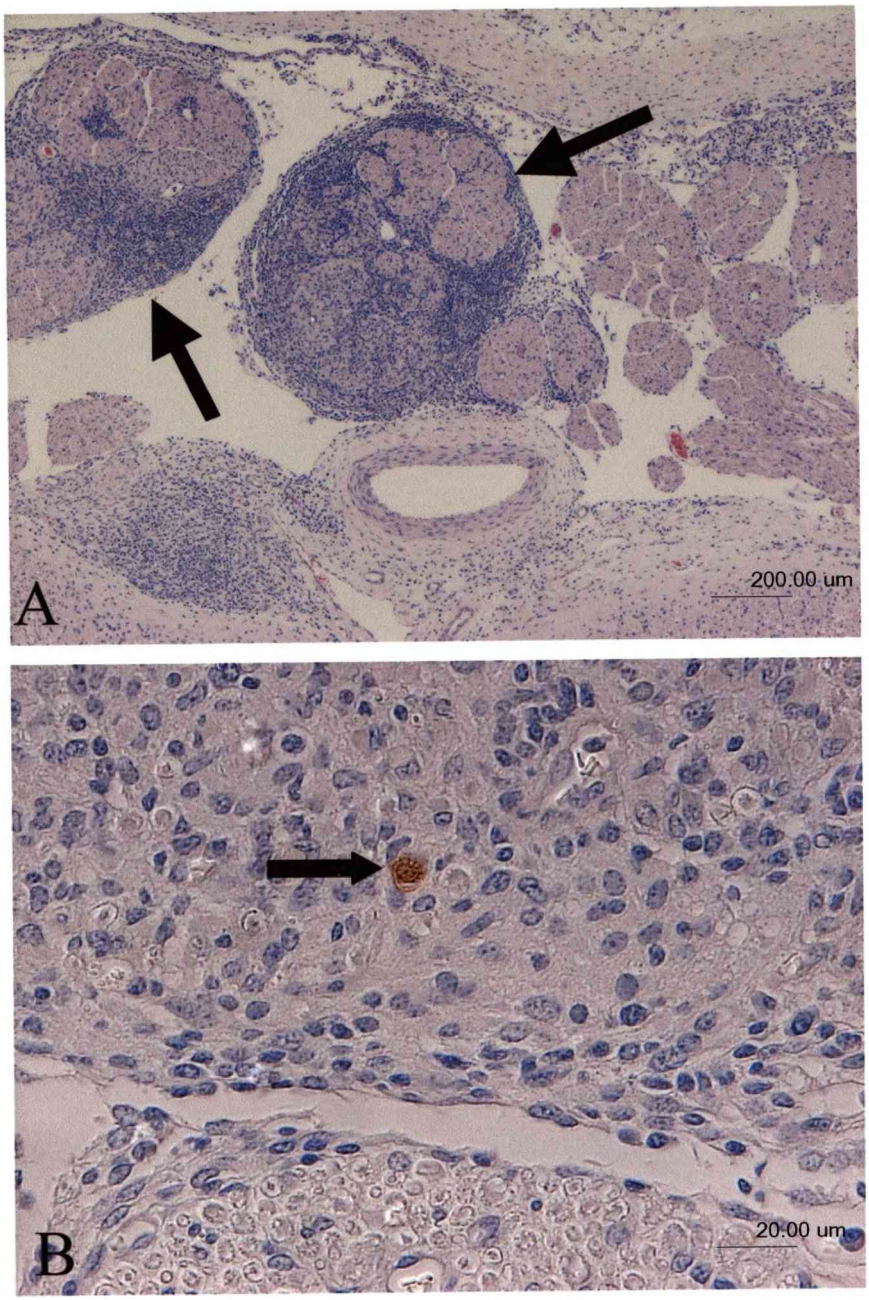



**Figure 4.4.7** Foetus 5 after recrudescence at 26 weeks gestation and necropsy at 31 weeks gestation. (A) Severe, multifocal bilateral non-suppurative radiculoneuritis (arrows), is seen. HE stain. (B) Immunohistology for *N. caninum* antigen identifies a small extracellular cluster of tachyzoites within the inflammatory infiltrates (arrow). PAP method, Papanicolaou's haematoxylin counterstain.

**4.4.4 The two calves born from chronically infected dams showed no histological abnormalities and parasites were not demonstrated immunohistologically in any tissues.**

Following euthanasia at two weeks of age, both calf 9 and calf 10 had no histological alterations in any of the tissues examined. Additionally, immunohistology for *N. caninum* antigen failed to detect parasites in any of the tissues from these animals.

**4.4.5 Parasite DNA is detected in 7/10 foetuses and is most commonly detected in the brain.**

From each foetus, brain, heart and liver were tested for the presence of *N. caninum* DNA. Parasite DNA was detected in all brain samples except those from foetuses 4, 7b and 8 (Table 4.4.3). Parasite DNA was detected in the heart in addition to the brain of foetus 5. In all other foetuses, parasite DNA was not detected in heart or liver.

In calves 9 and 10, no parasite DNA was detected in brain, heart or liver following euthanasia at 2 weeks of age.

**Table 4.4.3: Detection of N. caninum DNA in foetal tissues following recrudescence of infection in chronically infected cattle.**

| *Foetus number* | *Euthanasia (week of gestation)* | *Detection of parasite DNA* | | |
|---|---|---|---|---|
| | | *Brain* | *Heart* | *Liver* |
| *1a* | *21* | + | - | - |
| *1b* | *21* | + | - | - |
| *2* | *24* | + | - | - |
| *3* | *24* | + | - | - |
| *4* | *27* | - | - | - |
| *5* | *31* | + | + | - |
| *6* | *31* | + | - | - |
| *7a* | *34* | + | - | - |
| *7b* | *34* | - | - | - |
| *8* | *34* | - | - | - |

## 4.5 Discussion

We have compared the pathogenic effect of recrudescence of *N. caninum* in naturally, chronically infected cows at different stages of gestation by assessing the histological changes and the distribution of parasites in the placenta and foetal tissues.

Parasite recrudescence was associated with a sharp rise in *N. caninum* specific antibody levels in 9/10 animals between 20 and 37 weeks of gestation, with a gradual rise at the end of gestation evident in the remaining animal, which is in accordance with a previous study (Guy et al., 2001). In that study, foetal death occurred within one week of the antibody rise in one animal at week 17 of gestation. In our study foetal viability was monitored throughout pregnancy by ultrasonography and cows were euthanased after two successive antibody increases at weekly intervals, on the assumption that after that time, foetal death would not occur. This is supported by a further experimental infection with *N. caninum* tachyzoites at 70 days gestation, where foetuses died within 2 weeks of *N. caninum* specific maternal antibody levels reaching over the positive threshold (Macaldowie et al., 2004). Nonetheless, we have no guarantee that the foetuses would have survived to term.

It is worth considering that these animals were naturally infected cattle with presumably different strains of *N. caninum*, possibly with different virulence. It is unknown whether these animals had been infected in utero or post-natally via the ingestion of oocysts. This may have an effect on the efficiency of in utero

transmission in successive pregnancies. Some authors have suggested that post-natal exposure with oocysts is an inefficient route for establishing chronic infection (McCann et al, 2007). However other authors have suggested that animals infected post-natally can then transmit the parasite to the foetus in successive pregnancies (Dijkstra et al., 2008).

The fact that foetal death had not occurred within two weeks of parasite recrudescence in these animals agrees with other studies suggesting that, although foetal infection is common, foetal death occurs in only a minority of cases (Davison et al., 1999). In our study, the eight cows that were euthanased had evidence of placental necrosis, and 8/10 animals had at least one calf infected with *N. caninum*, as evident either by PCR, immunohistology or by the presence of *N. caninum* specific antibodies in the case of the two calves born at full term. In the remaining two animals in which parasites were not detected in the foetuses, one (animal 4) had parasites demonstrated in the placenta by immunohistology and the other (animal 8) had histopathological changes in the foetus indicative of neosporosis.

It could be argued in animal 4 that the parasite had not reached the foetus and was either being controlled by the immune response in the placenta or that the infection was not sufficiently well established to have reached the foetus in detectable numbers. In cow 5 the increase in antibody response was more gradual compared to the other cows and for this reason it was not euthanased until 31 weeks gestation, although the first increase in antibody was detected at week 26 of gestation. Parasite DNA was detected in the foetus from this cow in both the

brain and heart of the foetus, and it was one of only two foetuses that had parasites demonstrated immunohistologically. The most severe histopathological changes in both the foetus and placenta were detected in this animal.

Although all of the foetuses were alive at the time when the dams were euthanased, there was evidence of mild focal placental necrosis in all eight cows, with a mild, diffuse, lymphocyte-dominated, mononuclear interstitial infiltration. Parasites were detected sporadically within the areas of necrosis. Placental necrosis and inflammation has been described in field cases (Barr et al., 1990; Dubey and Lindsay 1996) and in our recent experimental infection detailed in chapter 3, of animals inoculated in parasites at both 70 and 210 days gestation where we saw extensive placental necrosis with foetal death after infection at 70 dg (Gibney et al., 2008).

Parasites were demonstrated either immunohistologically or by PCR in 7/10 foetuses. A drawback of this study is the fact that we do not have control tissues for these foetuses and therefore cannot rule out the possibility that the inflammatory processes seen could be due to some other factor apart from the parasite. However, none of the control tissues used for the foetuses in chapter 3 following infection at 210 dg had any histological changes. Following parasite recrudescence, histological alterations were detected in 7/10 foetuses, ranging from mild non-suppurative myocarditis and myositis to encephalitis and/or myelitis and radiculoneuritis, which was evident in most animals. Although encephalitis, myositis and myocarditis are regularly described in the literature as lesions commonly found in calves with neosporosis (Pescador et al., 2007),

myelitis is rarely reported. This is probably because the spinal cord is rarely examined in field cases of abortion rather than a true absence of lesions. The results of the present study suggest that the dorsal root ganglia are frequently involved in the inflammatory processes and represent a site of parasite seeding, as radiculoneuritis was detected in 3 foetuses. Radiculoneuritis is seen in neosporosis in dogs (Peters et al., 2000). The inflammation was lymphocyte dominated, although plasma cells and macrophages were also seen in the infiltrates, which is in agreement with other authors (Jardine and Last, 1993; Ogino et al., 1992). The finding of plasma cells gives some indication as to the duration of the inflammation, as they are not seen in inflammatory processes before day 5 (Messow and Hermanns, 1990). They were however, mainly seen in animal 5, which had a longer duration between recrudescence and necropsy (5 weeks) than the other animals.

There appeared to be no correlation between foetal age and the extent of inflammation in the foetus. Similar degrees of inflammatory lesions were detected in foetuses following parasite recrudescence at 20 and 33 weeks gestation. Should foetal immunocompetence be gradually developing at this time, you may expect to see more severe lesions in the more immature foetuses. This indicates a need for further studies looking at the parasite-specific immune response in the foetus.

The fact that these foetuses elicited a lymphocyte dominated inflammatory reaction adds more evidence to the theory that the immunocompetence of the foetus plays an important role in survival (Barr et al., 1994). It also demonstrates

that a limited inflammatory response does not have a major pathological impact on the foetus and its survival. The finding of parasite DNA most commonly in the central nervous system of the foetuses is also in agreement with other studies suggesting that the parasite has a predilection for nervous tissue in older foetuses (Collantes-Fernandez et al., 2006).

The two calves born at full term were serologically positive for *N. caninum*, but did not have histological alterations and no parasites were detected immunohistologically or by PCR In cow 9, a sharp rise in antibody was detected at week 37 of gestation and the calf was killed two weeks after birth, i.e. five weeks after the antibody surge. In cow 10, there appeared to be rising maternal antibody levels at week 40 of gestation. We cannot ascertain that this was when parasite recrudescence occurred in this animal. However, it is possible that detection of parasite recrudescence at the end of gestation could be masked by the fact that there is a shunt of immunoglobulins from the serum into the colostrum at this late stage of gestation (Williams et al., 2000). Parasites were not detected in either of these calves, suggesting that parasitaemia was controlled by the immune system. These calves are however born carrying infection, which is presumed to last for a lifetime. This suggests some form of immunological tolerance. The precise mechanism of how the immune response is sufficient to control parasitaemia, but allows the parasite to persist is unknown.

As discussed earlier in this thesis, the immune response to the parasite at the foetomaternal interface had been proposed as a potential abortifacient mechanism (Quinn et al., 2002). During pregnancy, the expression of T-helper

type 2 (Th2) and regulatory cytokines are thought to predominate in the placenta, and a Th1-type response, such as that seen following *N. caninum* infection, could be damaging to the pregnancy (Fried et al., 1998; Krishnan et al., 1996). In a parallel study involving the same animals, increased expression of both Th1 and Th2-type cytokines was observed in the placenta following recrudescence (Rosbottom et al., unpublished data). This accords with the experimental infection of cattle detailed in chapter 3, where a similar upregulation of both Th1 and Th2-type cytokines was observed (Rosbottom et al., 2008). It was suggested in chapter 3 that the greater upregulation of Th1 type cytokines (particularly IFNγ) following infection at 70 dg and leading to foetal death supports the theory that expression of proinflammatory cytokines at the foetomaternal interface is detrimental to pregnancy. Should this be the case, it is difficult to explain the upregulation of proinflammatory cytokines seen later in gestation and following recrudescence in chronically infected cows with the lack of foetal death. One theory is that down-stream immunoregulatory mechanisms such as expression of indoleamine 2,3 dioxygenase and heme oxygenase-1 may protect the placenta from the potentially harmful effects of proinflammatory cytokines (Munn et al., 1998; Zenclussen et al., 2005; Zenclussen et al., 2006).

This is the first study, to our knowledge, to look at the placenta and foetus following recrudescence in naturally, chronically infected cows, when the foetus had survived infection and was alive at the time of euthanasia. From our previous data we consider that it would be unlikely that foetal death would have occurred. The finding of parasite associated necrosis and inflammation in the placenta following natural recrudescence accords with the data presented in

chapter 3 of this thesis that suggests that parasites cross the placenta to infiltrate the foetal tissues rapidly after infection (Gibney et al., 2008). The mild focal necrosis and mild to moderate maternal interstitial inflammatory cell infiltration seen following recrudescence is similar to that seen following experimental infection at 210 dg, when the foetus survives (Gibney et al., 2008). In chapter 3, parasite load in the foetuses was much higher after infection at 70 days gestation than at 210 days gestation. The data presented here shows that, in naturally infected cattle carrying a chronic infection that recrudesced between these time-points, the parasite also crosses the placenta. Our data also suggests that lower parasite numbers were present in these foetuses compared to those experimentally infected at day 70 and are more similar to the parasite numbers seen following infection at 210 dg. These data support our hypothesis that in an older foetus there is less parasite associated pathological changes and necrosis and this may be due to a more mature foetal immune response.

The finding of parasite associated necrosis in the placenta and inflammation in the placenta and foetus in these cases has important implications for diagnosis. Firstly, it demonstrates that the parasite reached the foetus in 8/10 foetuses following recrudescence in the dam, suggesting that the maternal immune response to the parasite is not sufficient to prevent transmission to the foetus and that the immune response of the foetus is therefore more important in controlling parasite multiplication.

It also demonstrates that, in the case of aborted foetuses, the presence of parasites and inflammatory lesions within the foetus is not sufficient proof that the parasite

caused foetal death. As stated earlier, there are many other abortifacients in cattle and recrudescence of *N. caninum* could occur without fatal consequences at a similar time to another responsible infectious or non-infectious agent. This highlights the importance of a thorough abortion investigation in all cases of abortion in *N. caninum* seropositive cows.

# Chapter 5

# Concluding Discussion

The aim of this thesis was to address the question of how the parasite *N. caninum* causes abortion in cattle, based on the hypothesis that abortion occurs due to uncontrolled parasite spread in an immunologically immature foetus. This hypothesis was proposed since experimental studies have shown that the time of foetal infection during pregnancy is critical in determining foetal survival, with death more likely following infection early in gestation (Dubey et al., 1992; Barr et al., 1994; Williams et al., 2000). At these early stages of gestation the foetus is not able to respond fully to antigens (Osburn, 1986), suggesting that death may be the result of parasite multiplication in an immunologically immature foetus.

Accurate detection of the parasite in tissues is central to the investigation of this hypothesis. In chapter 2, the relative diagnostic sensitivity of two PCR protocols routinely used within our laboratory for the detection of *N. caninum* in tissues from aborted foetuses was determined, in order to use the more sensitive protocol for the experimental work presented. The nested PCR method of Uggla et al., (1998), based on the internal transcribed spacer 1 region of the rRNA sequence, was consistently more sensitive, detecting *N. caninum* DNA from brain samples seeded with 10 or more tachyzoites. This method was therefore used to analyse samples for the rest of the experiment. Another method used to detect the parasite in tissues was immunohistology, and a protocol was developed using a 3-step staining method incorporating an amplification product to increase sensitivity (peroxidase anti-peroxidase method; Kipar et al., 1998), details of which can be found in the methods section of chapter 3.

Having established sensitive methods to detect *Neospora* in tissues, two experiments were designed to investigate the thesis hypothesis. Chapter 3 describes the distribution of parasites and the histopathological changes in the placenta and foetus in 12 animals following experimental infection of cattle with *N. caninum* in early (day 70 of gestation (70 dg); n=6) and late (210 dg; n=6) gestation by PCR, light microscopy including immunohistology and transmission electron microscopy. The aim was then to verify the results from the experimentally infected cattle using ten cattle, naturally chronically infected with *N. caninum*. When the parasite recrudesced at different stages of pregnancy, the histological changes and distribution of the parasite in the placenta and foetus was assessed by PCR and light microscopy including immunohistology.

The experimental infections described in chapter 3 accord with a previous study (Williams et al., 2000), in that all foetuses died following infection early in gestation and survived following infection later in gestation. After infection at 70 dg, there was evidence in the foetal tissues of multifocal to coalescing necrosis associated with numerous parasites in all the organs examined. Despite the animals receiving the same dose of parasites at 210 dg, detection of parasites in the foetuses was restricted to the neural tissues – brain and spinal cord, with an associated mild focal encephalitis and myelitis. This suggests that the parasite is able to multiply in the younger foetuses to reach greater numbers, relative to the older foetuses who appear to be able to control parasite multiplication and hence survive. The fact that a mononuclear cell infiltrate was seen in areas of parasite-associated necrosis in the older foetuses but no inflammatory infiltrates were found in the younger foetuses supports the hypothesis that the older foetuses had

a sufficiently developed immune system that was capable of recognising the parasites as non-self and eliciting a lymphocyte dominated inflammation, which is consistent with the earlier work of Barr et al., (1994).

The differences we found in the placenta of the two groups of animals were also marked. After infection at 70 dg, extensive disseminated placental necrosis was seen, with tachyzoites in maternal and foetal epithelial cells and in leucocytes in placental maternal blood vessels. This is in contrast to after infection at 210 dg, where the placenta displayed mild multifocal necrosis, failure to demonstrate parasites by light microscopy (immunohistology) and only sporadic proof of the parasites by PCR. Additionally, after infection at 70 dg and 210 dg, a lymphocyte (predominantly CD4+ T cell) dominated mononuclear interstitial infiltration was seen in the placenta and the intensity increased with the extent of parasite-induced necrosis. This indicates a maternal immune response to the parasite. A previous hypothesis proposed was that this maternal immune response in the placenta may directly cause foetal death by eliciting necrotic changes in the placenta (Williams et al., 2000; Innes et al., 2005) but the data in chapter 3 suggests otherwise. It was proposed in the discussion of chapter 3 that the parasites rapidly passed across the placenta into the foetus in both groups and that the maternal immune response evident in both groups was controlling parasite multiplication in the placenta, but was not sufficient to prevent foetal infection. The changes in parasite load in the placenta were attributed to the differences in foetal immunocompetence. It was suggested in chapter 3 that after infection at 70 dg, parasites are able to multiply and re-invade the placenta via the foetal circulation, leading to extensive parasitaemia and necrosis of the

placenta. Conversely after infection at 210 dg, parasite multiplication is controlled by the foetal immune system and hence there is little evidence of re-invasion of the placenta.

The experiment described in chapter 4, studying parasite recrudescence in chronically infected animals, provided an additional insight into the pathogenic effect of *N. caninum* infection. Despite not causing foetal death in any of the 10 animals, we showed evidence that the parasite recrudesced between 20 and 37 weeks of gestation and passed through the placenta to the foetus in most cases. In 7/10 foetuses, parasites were detected either by immunohistology or PCR but were limited in their distribution. This contrasts with the situation seen in the cattle experimentally infected at 70 dg, and is a similar picture to that seen following experimental infection at 210 dg. Inflammatory processes were detected in 7/10 foetuses, including non-suppurative myocarditis, myositis and encephalitis and/or myelitis, as well as radiculoneuritis. These results add more weight to the theory that the immunocompetence of the foetus plays an important role in its survival, since all foetuses with lesions showed evidence of a localised lymphocyte-dominated inflammatory reaction to the parasite, and all foetuses survived.

Findings in the placentae of these animals also accord with the initial hypothesis, since placental damage was limited. There was evidence of mild focal placental necrosis in all animals, with a mild, diffuse, lymphocyte-dominated, mononuclear interstitial infiltration. Parasites were detected sporadically associated within the areas of necrosis.

The degree of placental necrosis and parasite numbers were far greater when foetal death occurred following infection at 70 dg and foetal death than following both infection at 210 dg and recrudescence in chronically infected cattle, when the foetus survived. The fact that the degree of placental necrosis correlates with numbers of parasites observed in the placenta confers with the theory that parasite mediated destruction of the placenta could be the cause of foetal death. Additionally, the detection of parasites in necrotic chorioallantoic villi strongly implicates them as the cause of necrosis.

The results of the experiments in this thesis raise questions over the diagnosis of *Neospora*-associated abortions (NAAs) in the field. It is commonly reported that the majority of NAAs are diagnosed at 3-8 months gestation, peaking at 4-5 months (Dubey et al., 2006), yet it is suggested from the results of this thesis that endogenous or exogenous transplacental transmission of *N. caninum* after 5 months of gestation does not result in foetal death, since in the naturally infected cattle, the first parasite recrudescence was detected at 20 weeks, and did not result in foetal death. Moreover all the foetuses experimentally infected at 210 dg (7 months) survived challenge.

Maley et al. (2003) carried out subcutaneous experimental infections in 14 cattle at 140 days gestation, followed by serial necropsies every 2 weeks from 14 to 56 dpi. Whilst it is not possible to ascertain that foetal death would have occurred in the foetuses necropsied early after infection since the experiment had not been carried out previously, it did appear that the placental necrosis and foetal CNS

lesions were resolving in the later necropsied animals. This experiment therefore suggests that transplacental transmission at this stage of pregnancy does not have a fatal outcome. However, since the infection was via the subcutaneous route it cannot be directly compared to our intravenous infections, especially since subcutaneous infection appears not to induce foetal death at 70 dg, a time when simultaneous intravenous infection resulted in 100% foetal death (Macaldowie et al., 2004).

Other authors have suggested that the development of a specific immune response may be variable from foetus to foetus (Adrianarivo et al., 2001). In dams inoculated with *N. caninum* between 159-169 dg, the foetuses, when removed for necropsy at 219-231 dg, showed strong humoral responses but highly variable CMI responses (Adrianarivo et al., 2001). In contrast, in cattle inoculated at 140 dg, mitogen responsiveness was demonstrated in foetal lymph node cells by 14 dpi, and specific cell proliferation and IFNγ responses from 28 dpi. Therefore the foetuses in this study did mount specific humoral and CMI responses following inoculation at mid gestation (Bartley et al., 2004).

A major drawback of this thesis is the fact that parasite-specific foetal immune responses were not directly measured. The studies in this thesis would have been enhanced by an analysis of the parasite specific immune response in the foetus, or at least an analysis of foetal fluids for antibody. Whilst the evidence collected in this thesis does support the fact that the older foetus appears able to control parasite multiplication, this could only be ascertained by further studies into foetal immunocompetence in cattle. Previous studies on how the foetus responds

to infection are scarce. Some studies have been carried out looking at adaptive foetal immune responses (Adrianarivo et al., 2001; Bartley et al., 2004), but knowledge of immune responses in general has progressed somewhat in recent years. Recent advances in studies of the innate immune system, an evolutionally conserved host defense mechanism against pathogens, would be an interesting point of further study. Areas to focus further investigation of foetal immune responses could include antimicrobial peptides and toll-like receptors, that are capable of sensing organisms including protozoa and play a major role in innate immunity (Uematsu and Akira, 2006). However, a method would need to be devised to study foetal immune responses whilst the foetus is alive in utero in a dam that is either chronically infected with *N. caninum* and monitored for recrudescence, or a cow experimentally infected with *N. caninum*.

In all animals, upregulation of Th1 and Th2-type cytokines was detected in the placenta, although a greater upregulation of a number of cytokines at the foetomaternal interface after infection at 70 dg was also detected in the experiment described in chapter 3 (Rosbottom et al., 2008). The cytokines that were upregulated included those implicated as potential abortifacients such as IFNγ (Kim et al., 2005; Krishnan et al., 1996), TNFα (Fest et al., 2006, Krishnan et al., 1996) and IL-2 (Tezabwala et al., 1989). It is possible that the high levels of these (particularly IFNγ) produced in the placenta following infection early in gestation could have direct cytotoxic effects and therefore contribute to foetal death. However, since these were also upregulated, albeit at lower levels, following infection at 210dg and following recrudescence in chronically infected

animals, when the foetuses survived, suggests that the placenta may not be as susceptible to these cytokines as has previously been proposed.

It is worth considering the pathogenesis of other bovine abortion agents in relation to neosporosis. In many cases, placentitis is attributed to be the cause of abortion. In *Brucella abortus,* the bacteria invade the placental trophoblasts and cause chronic placentitis, with foetal death being attributed to placental disruption and endotoxaemia. Similarly in *Listeria spp.*, foetal death is attributed to suppurative placentitis along with suppurative hepatitis in the foetus. A fibrinous, intercotyledonary placentitis and necrosis is described in *Campylobacter spp.,* (reviewed by Anderson, 2007). In these cases, the diagnosis of abortion is based on the demonstration of the agent in conjunction with a placentitis. The case is more complex for *N. caninum,* as we have demonstrated that the parasite can be detected in the placenta and/or foetus, causing a mild to moderate non-suppurative placental inflammation without causing foetal death. Another abortion agent with a more complex aetiology is Bovine virus diarrhoea (BVD) virus, in that this also can infect the foetus without a fatal consequence. BVD virus infections in the foetus can have variable outcomes depending on the gestational age of the foetus infected. Infection can lead to abortion at any stage of gestation, although it is more commonly seen in the earlier stages of gestation. Additionally, infection in the first 4 months of gestation can lead to persistently infected live calves. Mid gestational (100-150 day) infections can result in the birth of term calves with congenital abnormalities (reviewed by Grooms, 2004). The production of persistently infected calves in BVD virus differs from *N. caninum* in that they are only produced when the foetus is unable to recognise the

virus and is therefore immunotolerant. Persistently infected *N. caninum* calves do produce antibody, but harbour the parasite in a quiescent state, to be potentially reactivated in future pregnancies. However, whilst this is not true tolerance, this does suggest some form of latent infection. BVD virus also differs from *N. caninum* in that abortion can occur at any time, whereas the results of this thesis suggests that infection with *N. caninum* does not result in abortion from mid gestation onwards. Some authors have suggested an association between *N. caninum* and BVD virus. One study found a statistically significant association between presence of antibodies to *N. caninum* and BVDV in an aborting population, indicating that there might be concurrent effects of *N. caninum* and BVDV (Björkman et al., 2000). The relationship between *N. caninum* and other abortifacients in cattle is a potential area for future work.

In conclusion, the data presented in this thesis adds considerable weight to the hypothesis that the parasite *N. caninum* causes foetal death by multiplying uncontrollably in an immunologically immature foetus. Additionally, the thesis provides some interesting findings in the placenta and particularly the foetus following non-fatal endogenous or exogenous transplacental transmission. It is the first time parasites and histological alterations have been described in foetuses that would very likely have survived to term. These lesions (particularly non-suppurative encephalomyelitis and myocarditis) are similar to those lesions described in presumed cases of NAA and to which foetal death has previously been attributed. It therefore seems wise to end this thesis with a cautionary note for the diagnosis of NAAs. Based on the findings in this thesis, *N. caninum* should only be attributed to the cause of abortion if characteristic foetal lesions

are found in conjunction with a high maternal *N. caninum* specific antibody level and following a full investigation to rule out other causes of abortion. Even then, the fact that foetal infection occurs in most pregnancies in chronically infected cows, but abortion is only the outcome in a minority of cases should be borne in mind.

## APPENDIX 1 - RECIPES

### 1.1 Histology Reagents

Unless stated otherwise, all reagents are from VWR International, Lutterworth, UK

#### 4% neutral buffered paraformaldehyde

40g paraformaldehyde was dissolved in 800ml PBS (see appendix 1.3.2) at 60°C in a fume hood. The pH was adjusted to pH 7.24 - 7.4 and made up to 1 l with PBS. Solution was stored for up to one month.

#### Mayers haemalum

Haematoxylin 1gm
Sodium iodate 0.2gm
Potassium alum 50gm
Distilled water 1L

The haematoxylin and potassium alum was dissolved in distilled water using gentle heat, then allowed to cool. The sodium iodate was added and dissolved by shaking. The solution was then filtered and 20ml glacial acetic acid added.

#### Working solution of eosin

The stock solution of (1% w/v) was made by dissolving 1gm eosin (HD supplies, Aylesbury, UK) in 100ml distilled water

The working solution was prepared by mixing 50ml of 1% eosin with 390ml of 95% ethanol and 2ml glacial acetic acid.

## 1.2 Immunohistology Reagents

Unless otherwise stated, reagents are from VWR International, Lutterworth, UK

### Tris buffered saline (TBS, pH 7.6)

Tris(hydroxymethyl)methylamine 60.57g
Distilled water 610ml
1M HCl 390ml

The stock solution was made by mixing the ingredients above and adjusting the pH to 7.6. The working solution was made my adding 100ml of stock solution to 900ml of 0.8% NaCl.

### Phosphate buffered saline (PBS, pH 7.2)

NaCl 42g
$Na_2HPO_4.2H2O$ 9.26g
$KH_2PO_4$ 2.15g

These were dissolved in 1 l distilled water for the stock solution. The working solution was made by diluting the stock solution 1:5 in distilled water.

### 0.1M Imidazole buffer

0.1M imidazole stock was made by dissolving 6.81g in 1 l distilled water.
The buffer was made by adding 140ml 0.1M imidazole stock to 70ml 0.1M HCl.
The pH of the buffer was adjusted to 7.2.

### Papanicolaou's haematoxylin

Papanicolaou's haematoxylin was diluted 1:20 in distilled water and filtered before use.

### 10mM EDTA (pH 9)

0.372g EDTA was dissolved in 1 l distilled water and the pH adjusted to 9.0.

### Citrate buffer (pH 6)

Stock solution A (0.1M Citric acid)
2.1g citric acid in 100ml distilled water

Stock solution B (0.1M Sodium citrate)
14.7g tri-sodium citrate in 500ml distilled water

The working solution was made my mixing 9ml stock solution A and 41ml stock solution B with 450ml distilled water. The pH was adjusted to 6.

### 1.3 Electron microscopy reagents

All the reagents are from TAAB Laboratories, Aldermaston, UK

**Composition of resin mix for electron microscopy**

Resin (TAAB) 55g
Dodecenyl succinic anhydride 41g
Methyl nadic anhydride 13g
DMP30 [2, 4, 6, tri (dimethylaminomethyl) phenol] 2g

The first 3 components are thoroughly mixed before adding DMP30.

**EM fixative – 4% Paraformaldehyde + 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4).**

4g paraformaldehyde was dissolved in 30 ml distilled water by heating to 60°C. 1N NaOH was then added until the precipitate dissolved and was left to cool .10ml of 25% glutaraldehyde was added, and the solution made up to 50ml with distilled water. 50ml 0.2M sodium cacodylate (pH 7.4) was then added and the pH adjusted to 7.4.

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# The extent of parasite-associated necrosis in the placenta and foetal tissues of cattle following *Neospora caninum* infection in early and late gestation correlates with foetal death

E. Helen Gibney [a], Anja Kipar [b], Anne Rosbottom [a], Catherine S. Guy [a], Robert F. Smith [c], Udo Hetzel [b], Alexander J. Trees [a], Diana J.L. Williams [a,*]

[a] *Veterinary Parasitology, Liverpool School of Tropical Medicine/Faculty of Veterinary Science, Pembroke Place, Liverpool L3 5QA, UK*
[b] *Department of Veterinary Pathology, Faculty of Veterinary Science, University of Liverpool, Liverpool L69 7ZJ, UK*
[c] *Department of Veterinary Clinical Science, Veterinary Teaching Hospital, Leahurst, Neston CH64 7TE, UK*

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**Abstract**

The protozoan parasite *Neospora caninum* is the most frequently diagnosed abortifacient in the UK and a leading cause of abortion worldwide but the mechanisms leading to abortion are not fully understood. The distribution of parasites and the histopathological changes in the placenta and foetus were compared in 12 cows following experimental infection of cattle with *N. caninum* in early ($n = 6$) and late ($n = 6$) gestation, by PCR, immunohistology, light microscopy and transmission electron microscopy. Twelve uninfected pregnant cattle were used as controls. Infection in early gestation led to foetal death. In the placentae of cattle immediately following foetal death, *N. caninum* DNA was detected and there was evidence of widespread parasite dissemination. This was associated with extensive focal epithelial necrosis, serum leakage and moderate maternal interstitial mononuclear cell infiltration. In the foetuses, parasites were evident in all tissues examined and were associated with necrosis. In the placenta of cattle infected in late gestation, *N. caninum* DNA was detected sporadically but parasites were not evident immunohistologically. Small foci of necrosis were seen associated with mild interstitial mononuclear cell infiltration. Detection of *N. caninum* DNA in the foetuses was sporadic and parasites were demonstrated immunohistologically in brain and spinal cord only, with an associated mononuclear cell infiltration. This data is consistent with uncontrolled parasite spread in an immunologically immature foetus and could, via multiparenchymal necrosis of foetal tissues or the widespread necrosis and inflammation observed in the placenta, be the cause of *Neospora*-associated abortions.


*Keywords:* *Neospora caninum*; Cattle; Placenta; Foetal death; Necrosis; Inflammation

## 1. Introduction

The apicomplexan parasite *Neospora caninum* is the most frequently diagnosed abortifacient in dairy cattle in the UK and is a leading cause of abortion worldwide (Dubey, 1999; VLA Surveillance Report, 2006). Endogenous transplacental transmission, resulting from recrudescence of bradyzoite cysts in a chronically infected dam is the principle natural route of infection (Dubey and Lindsay, 1996). It has also been shown that cattle may abort after exogenous transplacental transmission, following ingestion during pregnancy of oocysts from the faeces of dogs, a definitive host (Gondim et al., 2004). However, although infection is common and transplacental spread of the parasite is highly efficient, only a proportion of infected cattle abort. The pathological processes leading to abortion in these animals are unclear (reviewed by Dubey et al., 2006). Experimental studies have shown that the time during pregnancy when foetal infection occurs is critical in determining foetal survival (Dubey et al., 1992;

* Corresponding author. Tel.: +44 151 705 3142; fax: +44 151 705 3373.
*E-mail address:* williadj@liv.ac.uk (D.J.L. Williams).

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Barr et al., 1994; Williams et al., 2000). Foetuses of cattle infected i.v. with tachyzoites at day 70 of gestation died 3–5 weeks p.i., whereas cattle infected at day 210 of gestation had pregnancies that continued to full term and gave birth to live, persistently infected calves (Williams et al., 2000). This finding was supported by a further study of naturally persistently infected animals, where foetal death occurred in one animal when the parasite recrudesced early in gestation, whereas in the remaining animals the parasite recrudesced later and the foetuses survived (Guy et al., 2001). It is clear that the timing of maternal parasitaemia, following either de novo infection or parasite recrudescence in persistently infected cattle, is crucial in determining the fate of the foetus.

Foetal immunocompetence starts to develop at 100 days gestation, but only after 150 days gestation is the foetus able to recognise and respond to antigens (Osburn, 1986), suggesting that a foetus would be more likely to survive if infected later in gestation whilst infection early in gestation may lead to foetal death as a direct result of parasite multiplication in an immunologically immature foetus. In support of this hypothesis, more protozoa were identified in foetuses less than 5 months old (Barr et al., 1994; Otter et al., 1995) and a higher number of PCR-positive tissue samples were observed in foetuses aborted due to *N. caninum* in the first and second trimester of pregnancy (Collantes-Fernández et al., 2006). Conversely, some authors have suggested that a foetal immune response to the parasite could in itself be damaging. Older foetuses showed a greater inflammatory response associated with the parasites (Barr et al., 1991). The most frequently observed lesions were multifocal non-suppurative encephalitis and myocarditis which were considered to be sufficient to cause foetal death (Wouda, 2000). Abortion could also occur as a direct result of parasite-induced damage to the placenta. In toxoplasmosis in sheep, it is considered that foetal death results from anoxia due to placental damage caused by the parasite (Buxton et al., 1982). In bovine neosporosis, focal necrosis and placentitis have been recorded both in field cases (Barr et al., 1991) and experimental cases (Barr et al., 1994; Maley et al., 2003; Macaldowie et al., 2004). Finally, it has been suggested that the maternal immune response recruited to control parasite-mediated cell damage in the placenta might be detrimental to foetal survival since the presence of cytotoxic T cells and pro-inflammatory cytokines in the uterus are thought to be incompatible with successful pregnancy (Raghupathy, 1997; Innes et al., 2002; Dubey et al., 2006).

It is clear that immunological control of the parasite in the placenta or by the foetus, either in a protective or detrimental role, could be the key to determining the mechanism of abortion. The aim of this study was to investigate the pathogenic processes which take place in the placenta and foetus in association with infection. We infected cattle with *N. caninum* in early and late gestation and compared the parasite distribution and pathological lesions in the placenta and foetus at a time when the foetus died, with a time where transplacental transmission occurred but the foetus survived.

## 2. Materials and methods

### 2.1. Source and husbandry of cattle

Twenty-four Holstein Friesian heifers aged 18–24 months were purchased from commercial farms and confirmed negative for evidence of exposure to *N. caninum* by antibody ELISA (Mastazyme, Mast Diagnostics, Merseyside, UK). Cattle were also confirmed negative for Bovine Viral Diarrhoea Virus (BVDV) antigen and vaccinated against *Leptospira* spp. (Leptavoid-H, Shering-Plough Animal Health, Uxbridge, UK), BVDV (Bovidec, Novartis Animal Health, Roysten, UK) and Infectious Bovine Rhinotracheitis (IBR) Virus (Bovilis IBR marker, Intervet, Milton Keynes, UK). Following testing and vaccination, cattle were housed in dog- and fox-proof accommodation. The cattle were oestrus synchronised using progesterone-releasing intra-uterine devices (PRID®, CEVA Animal Health, Watford, UK), artificially inseminated, then housed with a Hereford bull in dog- and fox-proof straw bedded pens. Pregnancy and foetal viability were confirmed at 35 days by trans-rectal ultrasonography.

### 2.2. Parasite infection and clinical monitoring of foetal viability

Cattle were divided randomly into two groups of 12. Half of each group ($n = 6$) received $10^7$ *N. caninum* Nc Liverpool strain tachyzoites in PBS, pH 7.2, i.v. at day 70 of gestation or at day 210 of gestation. Tachyzoites were grown in Vero cell cultures and purified using methods described previously (Williams et al., 1997). Briefly, tachyzoites were isolated by mechanically disrupting the cell monolayer through 21 gauge needles followed by passage over Sephadex G10 (PD10 columns, Pharmacia, Uppsala, Sweden) to remove Vero cell debris. The remaining half of each group ($n = 6$) were used as challenge controls and were inoculated with uninfected Vero cells treated the same way as those infected with tachyzoites. Foetal viability was monitored by transrectal (70 days gestation) or transabdominal (210 days gestation) ultrasound to detect a foetal heart-beat, twice-weekly until day 14 p.i. and daily thereafter (70 days gestation) or twice-weekly throughout (210 days gestation).

### 2.3. Analysis of peripheral immune responses

Blood samples were collected into heparinised and plain evacuated tubes (BD Ltd., Oxford, UK) before inoculation and then weekly for the duration of the experiment. Serum was collected by centrifugation at 1000*g* for 10 min and *N. caninum*-specific antibodies measured using the Mastazyme serum antibody ELISA (Mast Diagnostics). Peripheral blood mononuclear cells (PBMC) were isolated using

Lymphoprep (density 1.077 g/ml; Axis-Shield, Oslo, Norway) from heparinised blood and used to assay *N. caninum* antigen-specific proliferation using methods described previously (Williams et al., 2000). IFN-γ in cell culture supernatants was measured using the Bovigam IFN-γ ELISA kit (CSL, Victoria, Australia).

### 2.4. Euthanasia and sample collection

Euthanasia was carried out using a captive bolt pistol followed by pithing. After inoculation at 70 days gestation, the control group were euthanized 3 weeks p.i., at day 91 ± 1 of gestation, and the infected group within 24 h of detection of foetal death. All animals inoculated at 210 days gestation were euthanized 3 weeks p.i., at day 231 ± 1 of gestation. Immediately after euthanasia, the uterus was removed from each animal and the foetus recovered. For histology, 10 randomly selected placentomes with attached inter-placentome area and uterine wall were taken from each animal and the tissue fixed in 4% buffered paraformaldehyde (PFA; pH 7.4). Tissue samples were collected aseptically from all foetuses for storage at −20 °C for DNA extraction and for fixation in 4% PFA. They included the brain and spinal cord, an apical section of the heart, the lung, the main lobe of the liver, the spleen, the left kidney and adrenal gland, the pancreas, a section of jejunum, the quadriceps skeletal muscle, the femoral nerve, the umbilical cord, the femoral bone marrow, the thymus and mesenteric lymph node. In addition, samples of amniotic and allantoic fluid were collected in a sterile manner and stored at −20 °C for DNA extraction. Finally, small sections (approximately 2 $mm^3$) of placentome and foetal liver from each animal were fixed in 4% PFA with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for transmission electron microscopy (TEM).

### 2.5. Tissue processing for light microscopy

After fixation for up to 5 days, transverse sections of 10 placentomes including the attached inter-placentome area and uterine wall were prepared for each animal. The foetal brain was cut to obtain transverse sections of frontal cerebrum, occipital cerebrum and hippocampus, midbrain, cerebellum and medulla. Transverse sections were prepared from cervical, thoracic, lumbar and sacral areas of the spinal cord. Representative sections of all other tissues were prepared. Tissue sections were routinely embedded in paraffin wax. Consecutive sections (3–5 μm) were prepared and stained with H&E for histopathological examination or were used for immunohistology.

### 2.6. TEM

Based on the results from light microscopy, placenta and foetal liver from two representative animals infected at 70 days gestation were used for TEM. Fixed tissue samples were stored in 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4 °C until processing, post-fixed in 1% (w/v) osmium tetroxide and incubated in uranyl acetate before routine embedding in epoxyresin mix (TAAB Labs Eqpt., Ltd, Aldermaston, UK). Semi-thin sections (1.5 μm) were cut, stained with methylene blue and examined to select areas of interest (focal necrosis). From selected areas, ultrathin sections (75 nm) were prepared, stained with Reynold's lead citrate and viewed in an H-600 TEM (Hitachi, Pleasanton, California, USA).

### 2.7. PCR for N. caninum

From each foetus, a sample of brain, spinal cord, heart, liver, lung, kidney, skeletal muscle, amniotic and allantoic fluid was examined. Additionally, three samples each of placentome and interplacentomal area were examined from each placenta. From a 25-mg sample of solid tissue or 200 μl of fluid, DNA was extracted and purified with a DNeasy kit (Qiagen, Crawley, UK) and PCR reactions carried out using a modification of the nested PCR method of Uggla et al. (1998) with Hot star Taq (2.5 U per reaction) and buffer with a final concentration of 1.5 mM MgCl. Cultured *N. caninum* tachyzoite DNA and ultrapure water were used as positive and negative controls, respectively. PCR products were analysed by 2% agarose gels, stained with SYBR safe® DNA gel stain (Invitrogen, Paisley, UK) and visualised under UV light.

### 2.8. Immunohistology for N. caninum

Immunohistology for *N. caninum* was carried out on 3–5 μm sections consecutive to the H&E-stained sections. The peroxidase anti-peroxidase (PAP) method was applied as described (Kipar et al., 1998). Briefly, following dewaxing and rehydration, endogenous peroxidase was inactivated for 30 min in 0.5% hydrogen peroxide in methanol. Sections were incubated for 15–18 h at 4 °C with a polyclonal rabbit anti-*N. caninum* antibody (Barber et al., 1995), followed by 30 min incubation at room temperature with swine anti-rabbit IgG (DakoCytomation, Glostrup, Denmark) and rabbit PAP (DakoCytomation). Sections were washed in Tris-buffered saline (TBS, 0.1 M Tris–HCl with 0.9% NaCl, pH 7.2) between every incubation step. Visualisation was achieved by incubation for 10 min, stirring with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Fluka, Buchs, Switzerland) with 0.01% hydrogen peroxide in 0.1 M imidazole buffer, pH 7.1. Slides were counterstained for 30 s in Papanicolaou's haematoxylin and rinsed in running tap water before dehydrating, clearing and mounting.

Consecutive sections from each specimen were used as negative controls where the primary antibody was replaced with normal rabbit serum. A PFA-fixed, paraffin-wax embedded cell pellet of a tachyzoite infected Vero cell culture served as a positive control.

## 3. Results

### 3.1. Infection is established in all animals, but foetal death occurs only in those inoculated early in gestation

A viable infection was established in all animals inoculated with parasites. Infected animals seroconverted by 2 weeks p.i. and PBMC proliferated and secreted IFN-γ in response to *N. caninum* antigen from 1 week p.i. (data not shown). Seroconversion or proliferation was not observed in any of the control group animals. Foetal death occurred in all animals infected at 70 days gestation, 22.7 ± 1.2 days p.i. After infection at 210 days gestation, and in all control animals, foetuses were alive on the day of euthanasia (20–22 days p.i.).

### 3.2. Foetal death is associated with widespread placental necrosis and the presence of parasites within foetal and maternal epithelial cells

Histopathological examination of the placenta in animals where foetal death had occurred after infection at 70 days gestation revealed multifocal epithelial cell necrosis in all placentomes examined ($n = 10$ per animal). Both foetal and maternal epithelial cells were affected, ranging from single necrotic epithelial cells to extensive focal necrosis affecting large numbers of foetal villi and surrounding maternal epithelial cells (Fig. 1). Ultrastructural examination identified parasites within degenerating epithelial cells (Fig. 2A and B), extracellularly adjacent to areas of cellular debris (Fig. 2B) and extracellularly in the maternal interstitial layer. *Neospora caninum*-specific immunohistology showed structures resembling *N. caninum* tachyzoites and granular material, consistent with parasite debris or secreted antigen. Labelling was most intense within and adjacent to areas of necrosis, where antigen was observed both extracellularly and within degenerating cells. Tachyzoites were identified in the cytoplasm of intact and necrotic maternal endometrial and foetal trophoblast epithelial cells (Fig. 3A). Within necrotic areas and in the adjacent tissue, infiltration by macrophages and occasional neutrophils was observed (Fig. 3B). In addition, a moderate, diffuse, lymphocyte-dominated, mononuclear interstitial infiltration was present (Fig. 3C). At the base of the caruncles, mononuclear, lymphocyte-dominated perivascular infiltrates were observed. In most placentomes, focal haemorrhage and/or focal serum leakage was present at the villous tips or in the spaces between the maternal and foetal cell layers. Occasionally tachyzoites were identified by immunohistology within a circulating leucocyte in maternal blood vessels (Fig. 3D).

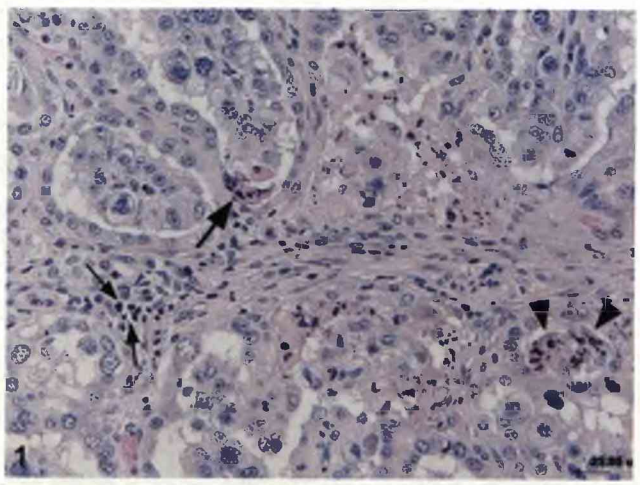

Fig. 1. Placenta after challenge with *Neospora caninum* at 70 days gestation when foetal death had occurred at day 26 p.i. Widespread epithelial necrosis is evident, affecting both maternal endometrial epithelium (large arrow) and foetal chorionic epithelium (arrow heads). A lymphocyte-dominated mononuclear infiltration is observed in the maternal interstitium (small arrows). H&E stain.

After infection at 210 days gestation, histological alterations were restricted to occasional, small areas of epithelial necrosis, usually affecting one foetal villus and the surrounding maternal epithelium. Where these lesions were present, a mild lymphocyte-dominated mononuclear interstitial infiltration was observed in the maternal tissue. *Neospora caninum* antigen was not detected by immunohistology.

In the two control groups, neither histological changes nor *N. caninum* antigen were detected in any of the placentomes examined.

### 3.3. Foetal death is associated with widespread necrosis in foetal tissues

Histopathological examination of foetuses that had died after infection at 70 days gestation revealed extensive necrosis in numerous foetal tissues. Necrosis was most severe in the brain, spinal cord (Fig. 4A) and liver (Fig. 4B) and was multifocal to coalescing. TEM examination of the liver confirmed that cell death of hepatocytes occurred via necrosis (Fig. 4C). In other tissues, scattered individual necrotic parenchymal cells were observed. These comprised myocytes in skeletal muscle, renal tubular epithelial cells as well as pulmonary and pancreatic parenchymal cells and cells in thymus, spleen and bone marrow. The remaining tissues (heart, adrenal gland, pancreas, jejunum, femoral nerve and mesenteric lymph node) did not exhibit any histological changes. There was no evidence of an inflammatory cell infiltration in any of the foetal tissues. Immunohistology identified *N. caninum* antigen in all foetuses and tachyzoites were detected in a number of intact and degenerating cells, in particular in glial cells in the brain and spinal cord (Fig. 4D), hepatocytes, skeletal myocytes, renal tubular epithelial cells and leucocytes in spleen and bone marrow. Tachyzoites were found within the cytoplasm of a megakaryocyte in the liver (Fig. 4E) and within the cytoplasm of haematopoietic cells in the bone marrow. Clusters of tachyzoites were also observed within single intact cardiac myocytes in six of seven foetuses (Fig. 4F).

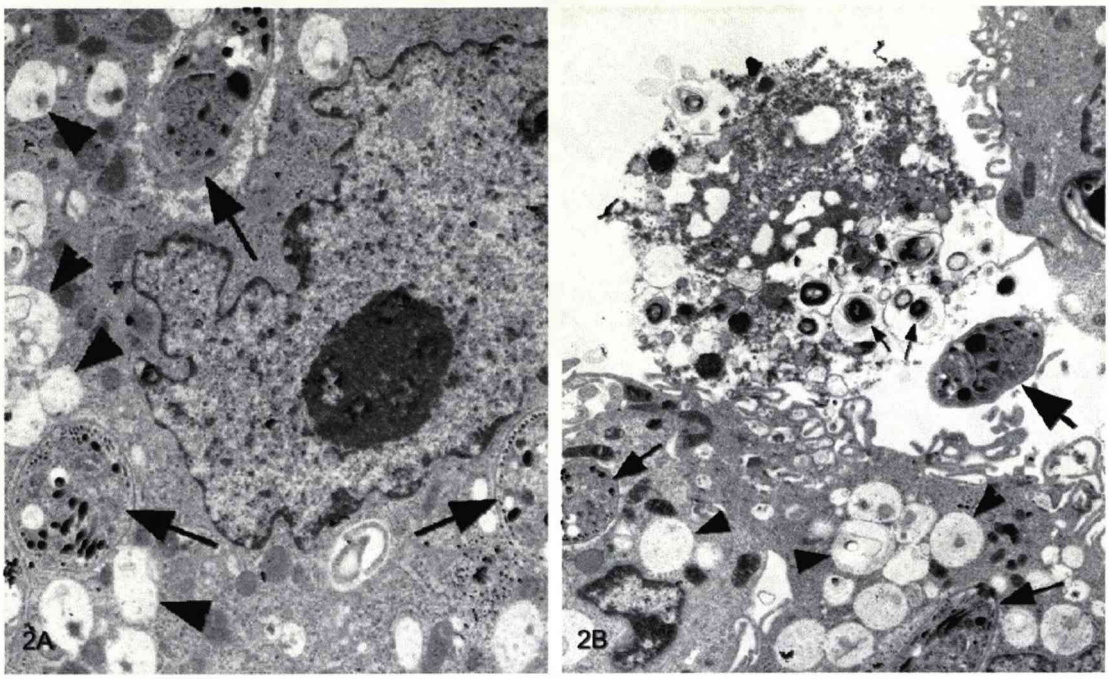


Fig. 2. Transmission electron micrographs of placenta after challenge with *Neospora caninum* at 70 days gestation when foetal death had occurred at day 19 p.i.. (A) Parasite-infected maternal epithelial cell. Three tachyzoites (arrows) are evident in the cytoplasm. The cell is exhibiting increased intracytoplasmic vacuolation (arrow heads) and mild nuclear protein margination (N, nucleus). (B) Two tachyzoites (arrows) in adjacent epithelial cells, exhibiting increased intracytoplasmic vacuolation (arrow heads). A third tachyzoite is cell-free (large arrow) adjacent to a cell that has undergone lysis and fragmentation (necrosis). Moderate sphingomyelin (small arrows) figure deposition (remnants of phagolysosomes) is evident.

After infection at 210 days gestation, histopathological changes were restricted to the foetal brain and spinal cord. In one foetus, focal microgliosis and perivascular mononuclear (lymphocytes and macrophages) cell infiltration in the spinal cord was observed (Fig. 5A) and in a second foetus focal encephalomyelitis with a perivascular mononuclear cell infiltration was seen. In both cases, *N. caninum* antigen was detected associated with these lesions (Fig. 5B). All other tissues examined from these two foetuses as well as all tissues from the other four foetuses were negative for parasite antigen.

In the two control groups, neither histological changes nor *N. caninum* antigen were observed in any foetal tissue.

### *3.4. Parasite DNA is widespread in placenta and tissues of foetuses that died as a result of infection but found in only a limited number of tissues from the placenta and foetuses of cattle infected at 210 days gestation*

Three samples each of placentome and interplacentome area were examined from the six animals infected at 70 days gestation. *Neospora caninum* DNA was detected in 17/18 of the placentomes and 7/18 of the interplacentome areas. Brain, spinal cord, lung, kidney and skeletal muscle were positive for *N. caninum* DNA in 7/7 foetuses, the liver in 6/7 and the myocardium in 5/7 foetuses. *Neospora caninum* DNA was detected in amniotic and allantoic fluid in 4/7 and 1/7 foetuses, respectively (Table 1).

In contrast, after infection at 210 days gestation, *N. caninum* DNA was only detected in 1/18 of the placentomes and 1/18 of the interplacentome areas. *Neospora caninum* DNA was detected in one tissue sample only from four of the six foetuses. The positive tissues were brain (1/6), skeletal muscle (1/6) and lung (2/6). *Neospora caninum* DNA was not detected in the remaining two foetuses (Table 1).

## 4. Discussion

We have compared the pathogenic effect of an i.v. infection of pregnant cows with *N. caninum* at an early (70 days gestation) and late (210 days gestation) stage of pregnancy by assessing the histological and ultrastructural changes in placenta and foetal tissues and the distribution of the parasite in tissues and cells. We used an i.v. inoculation of tachyzoites to represent a parasitaemia that could have resulted from either an endogenous or an exogenous infection.

Experimental i.v. inoculation resulted in placental and foetal infection regardless of the stage of gestation when the infection was administered, but there were substantial differences in the extent and pathological effects of infection. After infection at 70 days gestation, extensive, disseminated placental necrosis was seen. *Neospora caninum* tachyzoites were detected within both maternal and foetal epithelial cells, confirming the findings of others following experimental infection (Barr et al., 1994; Maley et al., 2003;

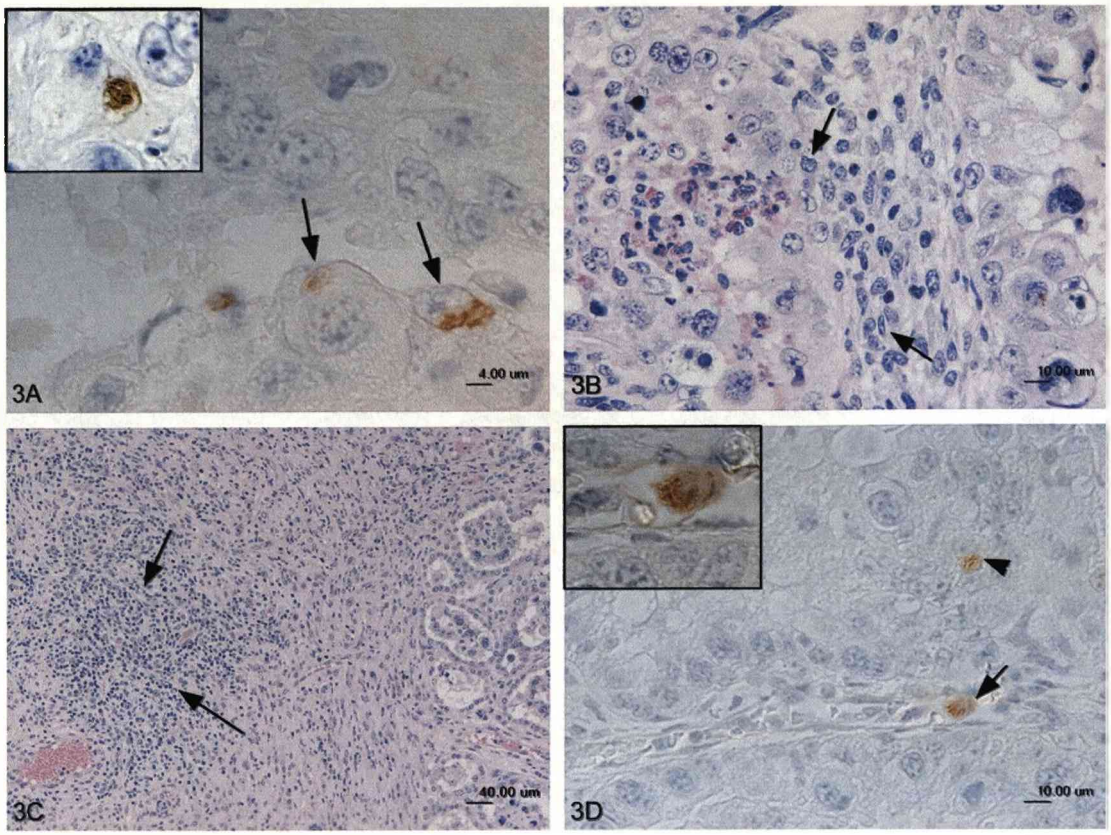


Fig. 3. Placenta after challenge with *Neospora caninum* at 70 days gestation when foetal death had occurred at days 26 (A–C) or 19 (D) p.i. (A) Immunohistology for *N. caninum* antigen identifies tachyzoites in foetal trophoblasts (arrows). (Inset) A cluster of tachyzoites is seen within a parasitophorous vacuole in the cytoplasm of an intact foetal trophoblast. PAP method, Papanicolaou's haematoxylin counterstain. (B) Macrophages are found associated with areas of necrosis and in the maternal tissue adjacent to these (arrows). H&E stain. (C) At the base of the caruncles, lymphocyte-dominated perivascular infiltrates are observed (arrows). H&E stain. (D) Blood vessel in the maternal placenta containing a maternal leucocyte with four tachyzoites in the cytoplasm (arrow; inset, higher magnification). In addition, a maternal epithelial cell containing tachyzoites is also present (arrowhead). Immunohistology for *N. caninum* antigen, PAP method, Papanicolaou's haematoxylin counterstain.

Macaldowie et al., 2004). We were able to demonstrate tachyzoites in leucocytes within placental maternal blood vessels, supporting the hypothesis that tachyzoites reach the placenta via the maternal circulation (Williams et al., 2000). Also, ultrastructural examination demonstrated parasites within the cytoplasm of foetal trophoblast cells undergoing necrosis.

In the foetuses there was multifocal to coalescing necrosis in numerous tissues. Moreover parasites were detected in a range of necrotic foetal parenchymal cells. In contrast cardiac myocytes appeared to undergo infection without cell death, suggesting that these may be the preferred cell type for the parasite.

In comparison, after infection at 210 days gestation, only very mild focal necrosis was seen in the placenta and evidence of *N. caninum* was only detected by PCR. Similarly whilst in five of six foetuses *N. caninum* was detected in foetal tissues either by PCR or immunohistology, changes in the foetuses were restricted to an occasional mild focal encephalitis and myelitis with the presence of parasite antigen in glial cells. This type of lesion has been reported in natural *Neospora*-associated abortions (Wouda, 2000) and yet the foetuses in this study were alive on the morning of euthanasia and, from our previous studies, would very likely have survived to term (Williams et al., 2000).

There was no evidence of inflammatory infiltrates associated with tissue necrosis in any of the foetal tissues at 70 days gestation whereas a mononuclear cell infiltrate was associated with areas of parasite-associated necrosis in foetal tissues at 210 days gestation. This supports the hypothesis that the older foetuses had a sufficiently developed immune system that was capable of recognising the parasites as non-self and eliciting a lymphocyte dominated inflammatory reaction. These findings are consistent with the work of Barr et al. (1994) who, although they used smaller numbers of cattle, were the first to suggest that a foetus's immunocompetence plays a pivotal role in its survival following infection with *N. caninum*.

The distribution of parasites in the foetuses which we describe here also agrees with a recent study on natural *Neospora*-associated abortion (Collantes-Fernández et al., 2006), in which parasite loads in the brain, heart, kidney and lung determined by real-time PCR were greatest in

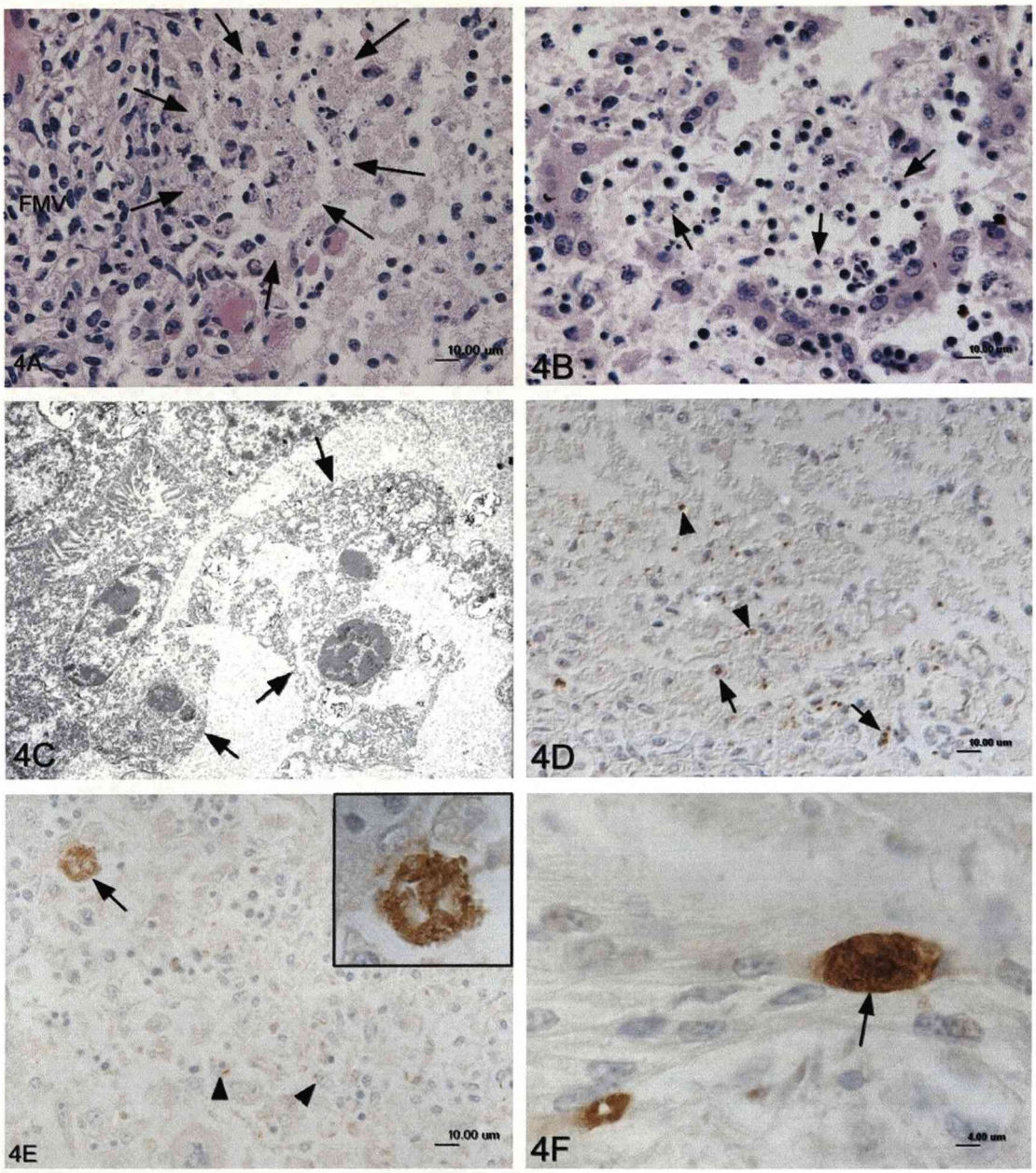


Fig. 4. Foetus after challenge with *Neospora caninum* at 70 days gestation when foetal death had occurred at days 26 (A–D and F) or day 19 (E) p.i. (A) Spinal cord, focal white matter necrosis (arrows). H&E stain. (B) Liver, multifocal hepatocellular necrosis (arrows). H&E stain. (C) Transmission electron micrograph of necrotic hepatocyte. Arrows point out an indistinct cell border and the loss of cytoplasmic and nuclear membranes. Karyorrhexis of the nucleus (N) is also evident. (D) Spinal cord, immunohistology for *N. caninum* antigen identifies scattered tachyzoites within glial cells (arrows) and extracellularly (arrowheads) within the area of necrosis. PAP method, Papanicolaou's haematoxylin counterstain. (E) Liver, immunohistology for *N. caninum* antigen identifies tachyzoites (arrows) within the cytoplasm of a megakaryocyte (inset, higher magnification) and within hepatocytes (arrowheads). PAP method, Papanicolaou's haematoxylin counterstain. (F) Heart, cluster of tachyzoites within an intact cardiac myocyte (arrow). Immunohistology for *N. caninum* antigen, PAP method, Papanicolaou's haematoxylin counterstain.

foetuses aborted in the first trimester. Foetal immunocompetence is thought to develop in cattle from around 100 days of gestation (Osburn, 1986). There are a limited number of studies that have investigated immune responses to *N. caninum* in an infected foetus. Antigen-specific IgG responses were detected in foetuses infected between 140 and 169 days gestation (Andrianarivo et al., 2001; Bartley et al., 2004) and antigen-specific cell proliferation and cytokine responses are detectable in most foetuses of this age but appear weak and variable (Almeria et al., 2003; Bartley et al., 2004). These results suggest that foetal immunocompetence is developing in mid-gestation but the rate at which the immune system develops and its ability to respond to a pathogen may vary from animal to animal.

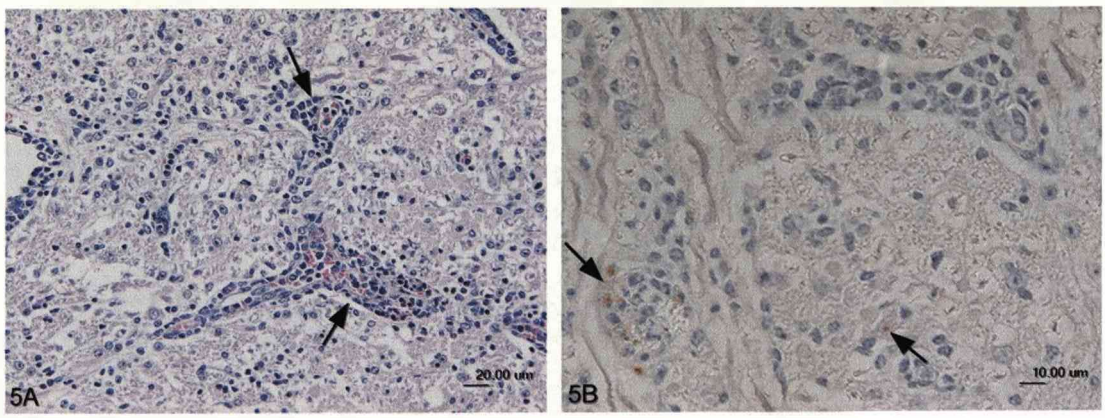


Fig. 5. Foetus 21 days after challenge with *Neospora caninum* at 210 days gestation when foetus was alive on morning of euthanasia. (A) Spinal cord. Focal microgliosis and perivascular mononuclear cell (lymphocytes and macrophages) infiltration (arrows). H&E stain. (B) Spinal cord. Immunohistology for *N. caninum* antigen identifies scattered tachyzoites (arrows) within the area of gliosis. PAP method, Papanicolaou's haematoxylin counterstain.

Table 1
Distribution of parasite DNA in placenta and foetal tissues after experimental infection with *Neospora caninum*, when foetal death occurs (70 days gestation) and when the foetus survives (210 days gestation)

| Tissue | 70 days gestation[a] | 210 days gestation[a] |
|---|---|---|
| Placentome | 17/18 | 1/18 |
| Interplacentome | 7/18 | 1/18 |
| Brain | 7/7 | 1/6 |
| Spinal cord | 7/7 | 0/6 |
| Liver | 6/7 | 0/6 |
| Heart | 5/7 | 0/6 |
| Lung | 7/7 | 2/6 |
| Kidney | 7/7 | 0/6 |
| Skeletal muscle | 7/7 | 1/6 |
| Amniotic fluid | 4/7 | 0/6 |
| Allantoic fluid | 1/7 | 0/6 |

Expressed as number of positive samples/number examined.
[a] Day of gestation that $10^7$ *N. caninum* tachyzoites were inoculated.

After infection at both 70 days gestation and 210 days gestation, a lymphocyte-dominated mononuclear cell interstitial infiltration was seen in the placenta, particularly in the maternal caruncle. The extent of this mononuclear infiltration varied with the extent of parasite-induced necrosis but indicates that the maternal immune system was actively responding to the parasite. We have shown elsewhere that the majority of these infiltrating lymphocytes were $CD4^+$ lymphocytes (Rosbottom et al., unpublished data) and this is consistent with other studies demonstrating the occurrence of $CD4^+$ T cells but also $CD8^+$ T cells, γδ T cells and natural killer (NK) cells in the placenta following infection at 70 days gestation (Maley et al., 2006). This maternal inflammatory cell infiltration as well as the widespread necrosis in the placenta has also been described in field cases (Barr et al., 1990; Dubey and Lindsay, 1996) and in cows experimentally infected at 70 days gestation (Macaldowie et al., 2004; Maley et al., 2006). Similarly, immunohistological demonstration of *N. caninum* tachyzoites associated with placental necrosis accords with previous studies (Macaldowie et al., 2004; Maley et al., 2006).

It has been suggested that the pro-inflammatory maternal immune response elicited in the placenta to control parasite multiplication and dissemination may be associated with life threatening necrotic changes in both placental and foetal tissues and is a direct cause of foetal death and abortion (Williams et al., 2000; Innes et al., 2005). However, the data we present here suggest that the foetal immune response may be more important in determining whether the foetus survives or is killed. In cattle infected at 70 days gestation all the foetuses died $22.7 \pm 1.2$ days later. However the same dose of parasites inoculated at 210 days gestation did not result in foetal death; the foetuses were alive on the morning of euthanasia and results of a previous study suggest that they would most likely have survived to term (Williams et al., 2000). Thus both groups of cows were infected with the same dose of parasites and killed at the same time after infection (approximately 21 days), yet there was evidence of much greater parasite load in the placenta of the cattle infected at 70 days gestation. It is likely that parasite multiplication in the caruncle is controlled by the maternal immune response which was evident in the placentas of cattle infected at 70 days gestation and those infected at 210 days gestation. This suggests that parasites had crossed the placenta and infiltrated foetal tissues rapidly after infection in both groups. Barr et al. (1994) suggested that tachyzoites cross the placenta and reach the foetus about 10 days after maternal infection. But in our study only in the day 70 group was there evidence of extensive parasite multiplication in the foetal tissues. This leads us to suggest that parasites multiply within tissues of the immunologically incompetent foetuses and then re-invade the placenta via the foetal circulation, leading to extensive parasitaemia of the placenta, particularly the cotyledons. In the foetuses of cattle infected at 210 days gestation few parasites were found, suggesting that parasite multiplication is controlled by the foetal immune response and as a result there is little or no re-invasion of the placental tissue. Others have shown parasites multiplying in the placenta and foetal tissues at 14 days p.i. (Macaldowie et al., 2004;

Maley et al., 2006) suggesting that parasites reach the placenta rapidly after i.v. inoculation. Parasites which had reached the placenta immediately after infection probably initiated the local maternal immune response, which we have shown involves up-regulation of pro-inflammatory, anti-inflammatory and regulatory cytokines (Rosbottom et al., unpublished data). This response may limit necrosis and placental changes in the first instance, suggesting that the maternal immune response may be beneficial to foetal survival but is unable to control the large numbers of parasites reinvading the placenta from the immunologically immature foetus.

Pinpointing exactly what causes *Neospora*-associated foetal death is difficult (Dubey et al., 2006). The results reported here showed that widespread parasite-associated necrosis of foetal tissues was evident, which may or may not cause foetal death depending on its extent. Equally, large areas of the placenta were damaged with evidence of parasites present within those necrotic areas, which could lead to placental insufficiency similar to that seen in toxoplasmosis where placental damage is considered to be the cause of foetal hypoxia and therefore death of the foetus (Buxton et al., 1982). Finally, the placentitis evident in these foetuses could be a further contributing factor.

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